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<b>14. ABSTRACT</b> During the reporting period continued progress has been made toward our original Specific Aims (1-3) through approved Modified Aims (M1 and M2) of our project, "Caffeine, adenosine receptors and estrogen in toxin models of Parkinson's disease (PD)". The overarching hypothesis of the project is that multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in PD. To that end we are characterizing the interplay between environmental agents (e.g., pesticides, caffeine, estrogen) that are leading candidate modulators of PD risk. In Year 5 we obtained and reported evidence that the adenosine receptor blocker caffeine as well as specific genetic depletion of the A2A subtype of adenosine receptor are capable of conferring protection against neuron degeneration in chronic pesticide and genetic mouse models of PD. Moreover, we have demonstrated and published that adenosine A2A receptors expressed in forebrain neuron are critical for neuron degeneration in toxin model of PD. We also identified CSF levels of urate (an environmentally and genetically determined antioxidant) is a predictor of progression of PD.					
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## Table of Contents

<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>7</b>
<b>Key Research Accomplishments.....</b>	<b>9</b>
<b>Reportable Outcomes.....</b>	<b>9</b>
<b>Conclusions/Modifications/Plans.....</b>	<b>10</b>

## Appendices

**A: Carta *et al*, 2009 [*J. Neurochem.*]**

**B: Ascherio *et al*, 2009 [*Arch. Neurol.*]**

**C: Carta *et al*, 2009 [*J. Neuropath. Exp. Neurol.*]**

**D: Kachroo *et al*, 2009 [*Soc. for Neurosci. abstract*]**

**E: Xiao *et al* [submitted manuscript]**

## **Introduction**

Identifying the mechanisms by which caffeine and more specific A<sub>2A</sub> antagonists protect dopaminergic neurons in multiple models of Parkinson's disease (PD) will advance our knowledge of the pathophysiology, epidemiology and therapeutics of PD.

The *overarching hypothesis* pursued by this proposal is that **multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease**. Here we endeavor to characterize the interplay between several environmental agents (pesticides, caffeine, estrogen and most recently the caffeine-related purine urate) that are leading candidate modulators of PD risk.

We are pursuing 3 *specific hypotheses*:

- 1) Caffeine acts through blockade of brain A<sub>2A</sub>Rs to protect dopaminergic neurons in both acute and chronic toxin models of PD. (*Specific Aim #1*)
- 2) Caffeine perfusion and focal A<sub>2A</sub>R inactivation within the striatum are sufficient to attenuate MPTP toxicity, by reducing toxin-induced release of glutamate and/or GABA. (*Specific Aim #2*)
- 3) Estrogen attenuates the protective effect of caffeine but not the protective of A<sub>2A</sub>R deletion because it acts by altering caffeine metabolism or A<sub>2A</sub>R expression. (*Specific Aim #3*)

## **Modifications for Yr 4-5**

**Specific Aim M1:** to definitively determine whether brain A<sub>2A</sub>Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD. [~280 mice]

**Hypothesis:** *Caffeine acts through blockade of brain A<sub>2A</sub> receptors to protect dopaminergic neurons in chronic pesticides (paraquat/maneb) model of PD.*

**Experiment #M1a:** **Effect of caffeine in Pq/Mb model using forebrain-neuron specific A<sub>2A</sub> KO mice.** Homozygous floxed A<sub>2A</sub>R littermates with or without *CAMKII Cre* (from matings of homozygous floxed A<sub>2A</sub> mice, one with Cre and the other without) will be treated chronically with caffeine or saline 10 min before paraquat and maneb or saline i.p. twice weekly for 8 weeks. Dopaminergic neuron degeneration will be measured one week after the last treatment by stereological cell counts of tyrosine hydroxylase-immunoreactive (TH-IR) cells in the substantia. Parallel assessment of striatal dopaminergic neuron injury will be assessed by <sup>3</sup>H-mazindol autoradiography for the striatal DAT binding. Glia (astrocytes and microglia) activation will also be measured using immunohistochemistry.

(Months 1-12)

**Experiment #M1b:** **Effect of caffeine in Pq/Mb model using astrocyte-directed A<sub>2A</sub> KO mice.** Homozygous floxed A<sub>2A</sub>R littermates with or without *GFAP Cre* will be treated with caffeine or saline followed by Pq/Mb (or saline/saline) injections biweekly for 8 weeks as in Exp #1. Genotyping and assessments of dopaminergic neuron injury and degeneration will be conducted as above.

(Months 6-18)

**Specific Aim M2:** to determine whether focal depletion of adenosine A<sub>2A</sub> receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity. [~360 mice]

**Hypothesis:** *Elimination of A<sub>2A</sub> receptors in striatum (but not substantia nigra or cortex) using a viral cre/loxP conditional knockout system will protect dopaminergic nigrostriatal neurons from MPTP toxicity.*

**Experiment #M2:** Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-cre) on MPTP-induced toxicity in floxed A<sub>2A</sub>R mice: Homozygous floxed A<sub>2A</sub>R mice (or wild type control mice) that previously received a stereotactic infusion of AAV-cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP. One week later infusion needle track will be localized histochemically, while dopaminergic neuron integrity will be visualized by striatal DAT binding and nigral dopaminergic neuron counts will be assessed as in Exp #4

**(Months 1-24:**

<b>striatal target:</b>	<b>months 1-12</b>
<b>nigral target:</b>	<b>months 7-18</b>
<b>cortical target:</b>	<b>months 13-24)</b>

## **Statement of Relevance** (from original proposal)

### **A. Parkinson's Disease -**

**Basic neuroscience significance** - The results will improve our understanding of adenosine receptor neurobiology, and will provide insight into the role of endogenous adenosine in basal ganglia biology physiology and PD pathophysiology.

**Epidemiological significance** - Establishing the ability of caffeine to protect dopaminergic neurons in PD models and identifying a plausible mechanism of action greatly strengthens the hypothesis that a neuroprotective effect of caffeine is the basis for its inverse epidemiological association with risk of PD.

**Therapeutic significance** - With several specific adenosine A<sub>2A</sub> antagonists emerging as promising therapeutic candidates based on their motor-enhancing (symptom-relieving) action, the prospects for additional neuroprotective benefit substantiated by this project may considerably enhance their therapeutic potential. In addition, identifying a biological basis for caffeine-estrogen interaction in modifying PD risk could also affect recommendations for estrogen replacement strategies in women with PD taking A<sub>2A</sub> antagonists or caffeine (and *vice versa*). Furthermore, based on evidence that A<sub>2A</sub>Rs contribute to the neurotoxicity affecting cortical and striatal neurons (as well as dopaminergic neurons), our findings may support novel A<sub>2A</sub>R-based neuroprotective treatments for a wider range of neurological diseases from stroke to amyotrophic lateral sclerosis (ALS) to Alzheimer's disease.

**B.Environmental Neurotoxin Exposure in Military Service** – By characterizing the neuroprotective effects of caffeine in a chronic pesticide model of PD (as well as the acute MPTP model), the proposed work will define a prototypical interaction between environmental toxins and protectants in determining the extent of a well-characterized neurological lesion (dopaminergic neuron death). Although there has been no compelling evidence to suggest that the incidence of PD will itself increase in association with military service or combat theatre exposures,<sup>[1]</sup> putative toxin exposure in the military may be linked to the development of another debilitating neurodegenerative disorder, ALS.<sup>[2]</sup> Moreover, some objective biological measures in veterans diagnosed with a “Persian Gulf War syndrome” have indicated dysfunction of dopaminergic neurotransmission in the basal ganglia,<sup>[3]</sup> raising the possibility (together with other data<sup>[4]</sup>) of altered risk for PD in this group. In any event, establishing a biological precedent for neurotoxin-neuroprotectant interplay in the relatively common disorder of PD, may provide a ‘roadmap’ that can be used should any neurological illness be confirmed to develop in association with prior military exposures.

**C.Understanding the Non-stimulant CNS Effects of Caffeine.** The psychoactive agent caffeine has been endorsed for military use at relatively high doses to help maintain operational readiness.<sup>[5]</sup> This recommendation has been based on a large body of evidence demonstrating sustainment of mental task performance by caffeine, and a lack of evidence for substantial harm at these doses. However, adopting the use of any CNS-active drug by protocol warrants careful consideration of newly appreciated neuronal actions of the agent. Accordingly, the proposed investigation of the novel neuroprotective effect of caffeine and its underlying mechanisms (e.g., altered neurotransmitter release) would be of significance for military programs that provide specific doses of caffeine to personnel to enhance cognitive function.

**D.Gender Differences in How Environmental Factors Impact Toxin Susceptibility.** Our investigation of how caffeine and estrogen exposures interact to modify neurotoxin susceptibility in laboratory models of PD may have substantial significance for the human epidemiology that prompted our pursuit of this line of research. In addition, the proposed studies may provide a prototype for modeling how gender and estrogen status interact with environmental exposures of relevance to the military (i.e., neurotoxins, caffeine). A better appreciation of how gender alters susceptibility to environmental toxins or protectants may ultimately lead to a better understanding (and modification) of the differential risks faced by women and men serving in the same military operations.

## **Body of the Report**

Progress during Year 5 on Specific Aims and experiments as laid out in our approved modification of the Statement of Work (SOW [in blue]) is described here in detail.

### **STATEMENT OF WORK** (focus on main areas of progress in Yr 5)

**Specific Aim M1:** to definitively determine whether brain A<sub>2A</sub>Rs (from either forebrain neurons or astrocytes) are required for caffeine's protective effect in chronic pesticides model of PD.

**[Please see manuscript and abstract publication in Appendix A.]**

- We have obtained and now published (Appendix A) evidence that forebrain A<sub>2A</sub> receptors are in fact required for dopaminergic neuron toxicity in a chronic toxin model of PD. Working with collaborators at the University of Cagliari we have compared the dopaminergic neuron toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine A<sub>2A</sub> receptor is conditionally 'knocked out' (cKO) of postnatal forebrain (striatal and cortical) **neurons**, compared to their control littermates with normal A<sub>2A</sub> receptor expression. The findings attenuated toxicity and inflammation support the possibility that specific adenosine A<sub>2A</sub> receptors -- currently in clinical (phase II/III) development as symptomatic therapy for later stage PD patients -- may have disease-modifying (neuroprotective) benefits if administered in earlier in the course of PD.

Using the same Cre/*loxP* cKO system, we similarly compared the toxicity produced by repeated daily administration of MPTP toxin in mice in which the adenosine A<sub>2A</sub> receptor is conditionally 'knocked out' of **astrocytes** compared to their control littermates with normal A<sub>2A</sub> receptor expression. The finding that these astrocytic A<sub>2A</sub> receptor cKO mice were not protected as observed in the neuronal A<sub>2A</sub> cKO, suggest neuronal A<sub>2A</sub> receptors could fully account for the hypothesized benefits of A<sub>2A</sub> antagonists against neurodegeneration in PD. The astrocyte A<sub>2A</sub> cKO data were removed from the final published manuscript pending a more complete validation of this cKO reagent (e.g., showing specific depletion of A<sub>2A</sub> receptors from astrocytes in this line, given that our findings with this line showed no phenotype in this PD model).

- Also in Year 5, and in pursuit of the original SA #1, we have completed studies (see our submitted manuscript in Appendix E) in the double adenosine A<sub>1</sub> and A<sub>2A</sub> receptor KO mice we developed under this project, in which the individual and combined roles of these receptors have been probed in a mouse model of levodopa-induced dyskinesia (LID) in PD. Because the model entails a unilateral lesion to dopaminergic nigrostriatal projection using the locally (intrastrially) applied neurotoxin 6-hydroxydopamine, an important determination was the extent of the lesion (assessed as the amount of striatal dopamine loss relative to the contralateral unlesioned striatum) in the presence or absence of either or both of these adenosine receptors. We report that using this toxin neither the single or double KOs altered the lesion. The demonstration that the receptors were not playing a critical role in this form of dopaminergic neuron injury, allowed for the

main interpretations of this experiment to focus on the role of the  $A_1$  and  $A_{2A}$  receptors in LID. Surprisingly, we found that the  $A_1$  as well as the  $A_{2A}$  receptor was required for the full development of LID in this mouse model of PD. This finding prompted to assess the effect of caffeine, a non-specific adenosine (mixed  $A_1$ - $A_{2A}$ ) receptor antagonist, and we found that like the  $A_1$ - $A_{2A}$  double KO, the mixed  $A_1$ - $A_{2A}$  antagonist also reduced LID. The result suggests that early adjunctive use of adenosine antagonists (including caffeine) with levodopa, could help prevent the development of disabling LID in patients with PD.

- Also in Year 5 we have further investigated and preliminarily reported (Appendix D) how depletion of adenosine  $A_{2A}$  receptors (in  $A_{2A}$  knockout mice) may mimic the neuroprotective effect of caffeine in another chronic (genetic) mouse model of PD. Here we used a lifelong exposure to an ‘endogenous toxin’ of transgenically expressed mutant human  $\alpha$ -synuclein (*hw- $\alpha$ SYN*). Mutations in the human  $\alpha$ -synuclein gene (A53T and A30P) have been associated with autosomal dominant familial PD. Transgenic mice expressing a human  $\alpha$ -synuclein gene with both of these mutations have been shown to develop age-related reductions in dopaminergic nigral neuron numbers, striatal dopamine (DA) and motor activity.

We investigated whether deletion of the  $A_{2A}$  receptor (in  $A_{2A}$ [-/-] mice) would afford protection against the age-related spontaneous reduction of striatal dopamine in the double-mutant human  $\alpha$ -synuclein (*hm<sup>2</sup>- $\alpha$ SYN*) mice. Heterozygous  $A_{2A}$ [+/-] mice were mated with  $A_{2A}$ [+/-] mice that were also transgenic for the wild-type (*hw- $\alpha$ SYN*) or *hm<sup>2</sup>- $\alpha$ SYN* form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase promoter – all in a congenic C57Bl/6 background. Offspring were sacrificed at 20-24 months of age and striatal DA and metabolites were measured by HPLC-ECD. As expected in the presence of a functional  $A_{2A}$  receptor, DA content was significantly lower (by 35%) in *hm<sup>2</sup>- $\alpha$ SYN* mice compared to either *hw- $\alpha$ SYN* or non-transgenic (NT) mice. By contrast, in  $A_{2A}$ [-/-] mice (lacking functional A receptor) the *hm<sup>2</sup>- $\alpha$ SYN* transgene did not reduce striatal DA levels compared to either control. Data for the dopamine metabolite DOPAC showed a similar trend, as did dopamine data when split out by gender.

In Yr 5, we completed stereological nigral TH-IR cell counts (Appendix D) clearly corroborating/confirming our observations with DA content above. Thus the  $A_{2A}$  receptor appears to be required for neurotoxicity in an  $\alpha$ -synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific  $A_{2A}$  antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

**Specific Aim M2:** to determine whether focal depletion of adenosine  $A_{2A}$  receptor in substantia nigra or striatum is sufficient to attenuate dopaminergic neuron toxicity.

**Effect of intracerebral infusion of Cre-expressing adeno-associated virus (AAV-Cre) on MPTP-induced toxicity in floxed  $A_{2A}R$  mice:** Homozygous floxed  $A_{2A}R$  mice that previously received a stereotactic infusion of AAV-Cre or AAV-green fluorescent protein (AAV-GFP) into the striatum (or frontal cortex or substantia nigra) will be acutely exposed to systemic MPTP...



Activity in Year 5 on SA M2 has primarily been in the expansion of our line of *floxed A<sub>2A</sub>* mice which is necessary to conduct the virally mediated focal eliminations of A<sub>2A</sub> receptors as planned. A period of reduced fertility/low offspring yields slowed the expansion. However after increasing the numbers of breeding pairs, the colony size has increased substantially in late 2009, with virally targeted Cre/*loxP* cKOs of A<sub>2A</sub> receptors in different brain regions now scheduled for January 2010. Other activity on the project in Year 5 has been the adoption in our laboratory of several measures of oxidative damage (nitrotyrosine and 4-hydroxy-2-nonenal [HNE], measure by immunohistochemistry or Western blotting) to be assessed in parallel with measures of dopanergic neuron injury and loss.

Also in Year 5 with this support of this award our laboratory has demonstrated that CSF levels of urate – a major antioxidant as well as the end product of adenosine metabolism in humans – along with those in serum are predictive clinical progression in patients with typical early PD, with publication of the work (Appendix B) seen as a major advance in PD research. These findings have helped accelerate development of a clinical trial (<http://clinicaltrials.gov/ct2/show/NCT00833690>) of inosine (deamination product of adenosine and precursor of urate) to test its safety as a means to elevate urate in PD ([http://www.michaeljfox.org/newsEvents\\_mjffInTheNews\\_pressReleases\\_article.cfm?ID=244](http://www.michaeljfox.org/newsEvents_mjffInTheNews_pressReleases_article.cfm?ID=244)).

### **Key Research Accomplishments** (in Year 5)

- Achievement of key components of Aim M1, in demonstrating/publishing that targeted depletion of forebrain A<sub>2A</sub> receptors reduces dopaminergic neuron degeneration in a sub-chronic toxin model of PD. The findings strengthen the translational rationale for conducting clinical trials with adenosine A<sub>2A</sub> antagonists (either caffeine or more specific blockers) as a potential neuroprotective strategy.
- Identification and publication that higher CSF levels of the adenosine end product urate as predictor of favorable outcomes in PD. The findings are being rapidly translated into a major new clinical trial targeting urate elevation as a novel neuroprotective strategy in PD.
- Completion of preparations to conduct the key experiments under Aim M2 in which virally delivered cre gene will produce a focal conditional knockout of A<sub>2A</sub> receptors to further localize the mechanism by which A<sub>2A</sub> antagonists can confer neuroprotection in PD models.

### **Reportable Outcomes**

#### 1) Publications (with acknowledgements citing W81XWH-04-1-0881/ USAMRAA)

- Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009) Inactivation of neuronal forebrain A<sub>2A</sub> receptors protects dopaminergic

neurons in a mouse model of Parkinson's disease. *J Neurochem.* Oct 8. [Epub ahead of print] [See **Appendix A.**]

- Ascherio A, LeWitt PA, Xu K, Eberly S, Watts A, Matson WR, Marras C, Kieburtz K, Rudolph, A, Bogdanov MB, Schwid SR, Tennis M, Tanner CM, Beal MF, Lang AE, Oakes D, Fahn S, Shoulson I, & Schwarzschild MA on behalf of the PSG DATATOP investigators. (2009) Urate as a predictor of rate of clinical decline in Parkinson disease. *Arch. Neurol.* (published online Oct 12, 2009). [See **Appendix B.**]
- McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ. (2009) Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. *J Neuropathol Exp Neurol.* 68:515-524. [See **Appendix C.**]
- Kachroo A, Richfield EK, Schwarzschild MA. 2009. Adenosine A2A receptor gene deletion confers protection of nigral neurons in an alpha-synuclein model of Parkinson's disease. 2008 Neuroscience Meeting. Chicago, IL: Society for Neuroscience. Abstract # 142.5. [See **Appendix D.**]

### **Conclusions/Modifications/Plans**

Central hypothesis: Multiple environmental protectants and toxins interact to influence of the health of the dopaminergic neurons lost in Parkinson's disease. Our progress under this award supports the central hypothesis, particularly with respect to caffeine-estrogen interactions in models of PD. In Year 6 we expect to focus on the completion of Aim M2 to localize the A<sub>2A</sub> receptors involved in dopaminergic neuron injury in models of PD. We also expect to expand the use of the conditional knockout reagents developed under this award to other environmentally relevant purine targets in PD, including adenosine metabolites like urate, which we have identified as a major new therapeutic target for neuroprotection in PD. Both our caffeine and urate findings hold promise for practical applications in the reduction of risk of developing PD or its decline.

Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009)  
 Inactivation of neuronal forebrain A2A receptors protects dopaminergic neurons in a  
 mouse model of Parkinson's disease. J Neurochem. Oct 8. [Epub ahead of print]



# Inactivation of neuronal forebrain A2A receptors protects dopaminergic neurons in a mouse model of Parkinson's disease.

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Date Submitted by the Author:	
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Keywords:	adenosine, MPTP, astroglia, microglia, tyrosine hydroxylase, substantia nigra

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Editor, Journal of Neurochemistry

Prof Tony Turner

Inactivation of neuronal forebrain A<sub>2A</sub> receptors protects dopaminergic neurons in a mouse model of Parkinson's disease.

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Adenosine A<sub>2A</sub> receptors antagonists produce neuroprotective effects in animal models of Parkinson's disease (PD). Since neuroinflammation is involved in PD pathogenesis, both neuronal and glial A<sub>2A</sub> receptors might participate to neuroprotection. We employed complementary pharmacologic and genetic approaches to A<sub>2A</sub> receptor inactivation, in a multiple 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, to investigate the cellular basis of neuroprotection by A<sub>2A</sub> antagonism. MPTP·HCl (20 mg/kg daily for 4 days) was administered in mice treated with the A<sub>2A</sub> antagonist SCH58261, or in conditional knockout mice lacking A<sub>2A</sub> receptors on forebrain neurons (fhnA<sub>2A</sub>KO mice). MPTP induced partial loss of dopamine neurons in substantia nigra pars-compacta (SNc) and striatum (Str), associated with increased astroglial and microglial immunoreactivity in these areas. Astroglia was similarly activated one, three and seven days after MPTP administration, whereas maximal microglial reactivity was detected on day one, returning to baseline seven days after MPTP administration. SCH58261 attenuated dopamine cell loss and gliosis in SNc and Str. Selective depletion of A<sub>2A</sub> receptors in fhnA<sub>2A</sub>KO mice completely prevented MPTP-induced dopamine neuron degeneration and gliosis in SNc, and partially counteracted gliosis in Str. Results provide evidence of a primary role played by neuronal A<sub>2A</sub> receptors in neuroprotective effects of A<sub>2A</sub> antagonists in a multiple MPTP injections model of PD. With the symptomatic antiparkinsonian potential of several A<sub>2A</sub> receptor antagonists being pursued in clinical trials, the present study adds to the rationale for broader clinical benefit and use of these drugs early in the treatment of PD.

Keywords: adenosine, MPTP, microglia, astroglia, tyrosine hydroxylase

Abbreviated title: Neuronal A<sub>2A</sub> receptors and neuroprotection

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Since the initial demonstration that caffeine can attenuate MPTP-induced toxicity, a neuroprotective role of A<sub>2A</sub> receptor blockade has been suggested in several experimental models of Parkinson's disease (PD) (Chen et al., 2001; Schwarzschild et al., 2006). In rodents different A<sub>2A</sub> receptor antagonists have been shown to counteract the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), as well as dopamine depletion in the striatum (Str), induced by acute administration of systemic MPTP or acute infusion of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (Chen et al., 2001; 2002; Ikeda et al., 2002; Pierri et al., 2005). Moreover, loss of striatal dopamine induced by acute MPTP was attenuated by the global genetic deletion of A<sub>2A</sub> receptors in A<sub>2A</sub> knockout (KO) mice (Chen et al., 2001). In contrast to acute models, neuroprotection by A<sub>2A</sub> antagonists in the neurotoxicity induced by multiple MPTP administration was never evaluated. On the other hand, previous studies have shown that acute or multiple injections MPTP delivery result in different histopathological features and different modes of cell death (Schmidt and Fergen, 2001). Interestingly, A<sub>2A</sub> receptor antagonism or gene KO has been found to be neuroprotective in different models of neurodegeneration, such as Alzheimer's disease, Huntington's disease and cerebral ischemia (Chen et al., 1999; Dall'Igna et al., 2003; Fink et al., 2004; Melani et al., 2003).

A<sub>2A</sub> receptors are enriched in the Str, where they are located either postsynaptically in striatopallidal neurons, or presynaptically in nerve terminals (Rebola et al., 2005; Rosin et al., 1998; Schiffman et al., 1991; Svenningsson et al., 1999). Moreover, A<sub>2A</sub> receptors are expressed at low level in other forebrain structures, such as cortex and hippocampus, whereas little or no A<sub>2A</sub> receptor immunoreactivity has been detected in dopaminergic neurons in the SNc (Rosin et al., 1998). Besides neurons, non-neuronal cell types such as microglia and astroglia also express A<sub>2A</sub> receptors (Fiebich et al., 1996; Saura et al., 2005).

The mechanism through which A<sub>2A</sub> receptor antagonists achieve neuroprotection in PD models has not been elucidated. Given the cellular distribution of A<sub>2A</sub> receptors in different cell types in brain,

neuroprotection by A<sub>2A</sub> receptor blockade may be achieved through an action on receptors located either on neurons or on glial cells. Attenuation of gliosis by A<sub>2A</sub> receptor antagonists in the Str and SNc of mice acutely treated with MPTP, suggests that reduction of neuroinflammation may be involved (Ikeda et al., 2002; Pierri et al., 2005). Moreover, it was recently suggested that A<sub>2A</sub> receptors located on glial cells may play a role in neuroprotection mediated by A<sub>2A</sub> antagonists against an acute MPTP-induced striatal dopamine depletion (Yu et al, 2008).

In order to get insight into the mechanism by which A<sub>2A</sub> receptor antagonists induce neuroprotection, the present study evaluated 1) the neuroprotective activity of an A<sub>2A</sub> antagonist in a mouse model of PD obtained by a multiple injections MPTP delivery; 2) the role of neuronal A<sub>2A</sub> receptors on this effect and 3) whether neuronal A<sub>2A</sub> receptor blockade might affect glial response to MPTP. To this aim, we treated mice with MPTP plus the A<sub>2A</sub> antagonist SCH58261, or administered MPTP to genetically manipulated mice selectively lacking A<sub>2A</sub> receptors in forebrain neurons (fbnA<sub>2A</sub>KO mice) (Bastia et al., 2005; Shen et al., 2008). Neuronal damage was evaluated in the SNc and Str using tyrosine hydroxylase (TH) immunohistochemistry, whereas the inflammatory response in the SNc and Str was studied through analysis of glial fibrillary acidic protein (GFAP) and CD11b immunoreactivity as markers of astroglial and microglial cells, respectively.

The present study provides evidence of a primary role played by forebrain A<sub>2A</sub> receptors in the neuroprotection that A<sub>2A</sub> receptor antagonists confer against dopaminergic neuron degeneration and glial activation induced by repeated MPTP.

## Methods

*Animals and treatments:* Male C57BL/6J mice (Charles River, Italy) were used for experiments involving pharmacological treatments with the A<sub>2A</sub> receptor antagonist. Mice were housed 5 per cage,

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3 with a 12:12 hrs light/dark cycle and with food and water *ad libitum*. Experiments were conducted in  
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6 accordance with the guidelines for care and use of experimental animals of the European Communities  
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8 Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health.

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11 Pharmacological treatment: Male C57BL/6J mice (25-30 gm, 3 months old), received a multiple  
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13 injections treatment with vehicle (N= 15), MPTP·HCl (20 mg/kg i.p.) once a day for 4 days (N = 18),  
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15 or the A<sub>2A</sub> antagonist SCH58261 (0.5 mg/kg i.p.) twice a day plus MPTP (20 mg/kg i.p.) once a day for  
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17 4 days (N = 23). SCH58261 was injected half an hour before MPTP administration. After MPTP  
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19 treatment discontinuation SCH58261 treatment continued once a day, until sacrifice, which occurred 1,  
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21 3 or 7 days after MPTP treatment. Injections were made at 8 a.m. and 8 p.m.

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26 Postnatal forebrain neuron conditional A<sub>2A</sub>KO mice were generated using the Cre/*loxP* system based  
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28 on the specificity of *CaMKIIα* promoter (Bastia et al, 2005, Yu et al, 2008; see also Additional  
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30 Informations (A.I.) for online version). FbnA<sub>2A</sub>KO mice and littermate controls were treated with  
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32 repeated MPTP (n=18 or n=15) or vehicle (n=12 or n=10) as described above. Experiments with the  
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34 A<sub>2A</sub> antagonist (see below) showed that loss of TH-immunoreactive cells was stable 1, 3 and 7 days  
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36 after MPTP treatment, and that CD11b immunoreactivity was greatest 1 and 3 days post-MPTP  
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38 treatment. Based on these considerations, neuroprotection and glial reactivity parameters in transgenic  
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40 mice were evaluated 3 days after MPTP treatment.

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46 *Drugs*: MPTP·HCl (Sigma-Aldrich, Italy or USA) was dissolved in 0.9% saline in a volume of 0.1  
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48 ml/10 g; SCH58261 (kindly provided Prof Baraldi, Ferrara) was suspended in 0.5% of methylcellulose.

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51 *Immunohistochemistry*: Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) or Avertin  
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53 (0.1 ml/10 g i.p.) prior to transcardial perfusion with 20 ml of saline and 60 ml of 4%  
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55 paraformaldehyde. Brains were removed and post-fixed for 2 hour. Adjacent coronal sections (50 μm)  
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57 from the Str and SNc were cut on a vibratome and stored at -20°C in an antifreeze medium until use  
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(Schintu et al, 2009). For TH, GFAP and CD11b immunostaining, adjacent sections were processed as described (Schintu et al, 2009; See also A.I.).

*Analysis and statistics:* Images were digitized (videocamera Pixelink PL-A686) under constant light conditions to standardize the measurements. Immunostained sections containing left and right SNc, were captured at 10X magnification (the entire SNc, corresponding to three frames, was digitized for the analysis). Immunostained sections of bilateral striata were captured at 20X magnification. One portion from the dorsolateral Str and one from the ventromedial Str (520  $\mu\text{m}$  x 380  $\mu\text{m}$ ) were analysed. For each animal, three sections corresponding to rostral (within -2.90/-3.20 mm from bregma), medial (-3.20/-3.50) and caudal (-3.50/-3.80) SNc levels, and three sections corresponding to rostral (within 1.20/0.90 mm from bregma), medial (0.90/0.60) and caudal (0.60/0.30) Str (accordingly to Mouse Brain Atlas, Paxinos and Franklin, 2001) were analysed for each protein marker evaluated in the study. *TH and GFAP analyses:* since the number of cells was different in the three SNc and Str levels analysed, for each mouse the number of TH- or GFAP-positive cells/level was first normalized with respect to the vehicle. Individual values from the 3 levels were then averaged to generate a mean. Adjacent SNc sections were stained with cresyl violet, in order to confirm cell loss in this area. The SNc from three mice randomly selected from each experimental group were evaluated by unbiased stereological counting and the mean of TH-ir neurons per  $\text{mm}^3 \pm \text{SEM}$  was calculated (see A.I.).

*CD11b analysis:* images were digitized in a grey scale, and CD11b immunostaining was evaluated with the analysis program Scion Image. A threshold, the value of which was set above the mean value  $\pm$  S.E.M. of the background, was applied for background-correction. Inside each frame, the area occupied by grey values above the threshold was automatically calculated. For each level of SNc or Str, the

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3 obtained value was first normalized with respect to vehicle; the individual values from the 3 levels  
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6 were then averaged to generate a mean.  
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9 Results from mice treated with MPTP plus SCH58261, or from fbnA<sub>2A</sub>KO mice were statistically  
10 compared with a two-factors ANOVA, followed by Tukey's *post hoc* test, for comparison between  
11 experimental groups.  
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19 **Results**

20 **A<sub>2A</sub> receptor antagonist prevents dopaminergic cell loss in the SNc.**

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24 In C57BL/6J mice, MPTP treatment induced a partial dopaminergic neurons degeneration in the SNc  
25 (Fig 1A, B and Tab 1). Counting of cresyl violet stained cells confirmed this result (Fig 1A, Vehicle:  
26 100 ± 1.5% versus MPTP: 67.8 ± 4.3%; as measured three days after last injection, relative to vehicle  
27 controls). Dopaminergic neurons loss was statistically significant 1 day after MPTP treatment (N= 5)  
28 and remained significant after 3 (N=5) and 7 days timepoints (N=8). Combined treatment with A<sub>2A</sub>  
29 antagonist SCH58261 plus MPTP, attenuated TH-positive neurons loss in the SNc at 1 (N= 5), 3 (N=5)  
30  
31 and 7 days (N=13) (Fig 1 A, B and Tab 1), as confirmed by cresyl violet staining (MPTP + SCH58261:  
32 98.6 ± 1.3%). Two-factors ANOVA showed a significant effect of treatment (see Table 2 in A.I. for  
33 corresponding F and P values). In the Str, MPTP-induced decrease in TH-immunoreactivity was  
34 significantly attenuated by treatment with the A<sub>2A</sub>R antagonist, as measured three days after treatment  
35 (Fig 2).  
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52 **A<sub>2A</sub> receptor antagonist inhibits astroglia and microglia activation.**

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3 In brain sections from vehicle-treated mice, few GFAP-positive cells (Fig 3), and a low CD11b  
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5 immunostaining (Figs 3, 4), were detected in the SNc and Str. Repeated MPTP treatment induced an  
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7 increase in GFAP and CD11b-positive cells in both the SNc and Str of C57BL/6J mice.  
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10 GFAP-positive cells displayed a highly branched morphology with tiny processes and a small body in  
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12 control sections, and became hypertrophic in response to MPTP treatment (Fig 3A); moreover, CD11b-  
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14 positive cells were ramified at baseline, but took on an ameboid aspect after MPTP treatment (Fig 4A),  
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16 indicative of astroglial and microglial activation. In both the SNc and Str, GFAP immunolabelling was  
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18 of similar intensity 1, 3 and 7 days post-MPTP treatment (Fig 3B). Combined SCH58261 plus MPTP  
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20 treatment, completely prevented the increase in GFAP immunoreactivity in the SNc and partially  
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22 prevented it in the Str, at 1, 3 and 7 days after MPTP treatment (Fig 3B). Two-factors ANOVA for  
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24 GFAP analysis showed a significant effect of the treatment at all time points analyzed in the SNc and in  
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26 the Str (see Table 2 in A.I.).  
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31 CD11b immunolabelling was highest 1 day after MPTP, gradually declining to basal levels after 7 days  
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33 in both SNc and Str (Fig 4A, B). Combined SCH58261 plus MPTP treatment completely prevented the  
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35 increase in CD11b immunoreactivity in the SNc 1 and 3 days after MPTP treatment (Fig 4A,B). In the  
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37 Str, SCH58261 partially prevented MPTP-induced increase in CD11b at 1 day, and totally prevented it  
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39 at 3 days after MPTP treatment. Two-factors ANOVA and *post hoc* analysis for CD11b revealed a  
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41 significant effect of treatment, time, and a treatment/time interaction, 1 and 3 days after MPTP  
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43 administration in the SNc and in the Str (se Table 2 in A.I.).  
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### 51 **Dopaminergic cell loss is prevented in fbnA<sub>2A</sub>KO mice.**

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53 In fbnA<sub>2A</sub>WT mice, repeated MPTP treatment induced a significant loss of dopamine neurons in the  
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55 SNc (Fig 5A, B and Tab 1). This result was confirmed by a similar reduction in cresyl violet stained  
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57 cells (fbnWT V:  $100 \pm 1.21$ ; fbnWT MPTP:  $71.60 \pm 2.73$ ) (see right insert in fig 5A). In contrast, in  
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fbnA<sub>2A</sub>KO mice, repeated MPTP treatment did not result in a decrease in TH-immunolabelling in the SNc, as confirmed by analysis of cresyl violet staining (fbnA<sub>2A</sub>KO V: 99.12 ± 1.41; fbnA<sub>2A</sub>KO MPTP: 99.71 ± 4.40) (Fig 5A, B and Tab 1). Two-factors ANOVA revealed a significant effect of treatment and genotype (see Table 2 in A.I. for corresponding F and P values). In the Str of fbnA<sub>2A</sub>KO mice repeated MPTP reduced TH immunolabelling to a lesser extent as compared with fbnA<sub>2A</sub>WT controls (Fig 2).

**Astroglial and microglial activation is attenuated in fbnA<sub>2A</sub>KO mice.**

GFAP immunoreactivity in the SNc and Str was significantly enhanced in MPTP-treated fbnA<sub>2A</sub>WT mice (Fig 6A, B). In contrast, in fbnA<sub>2A</sub>KO mice, MPTP-induced increase in GFAP immunoreactivity was totally prevented in the SNc, and partially prevented in Str (Fig 6A, B). In both brain regions two-factors ANOVA revealed a significant effect of treatment, genotype and a significant treatment/genotype interaction (see Table 2 in A.I.).

CD11b immunolabelling in the SNc and Str was significantly enhanced in MPTP-treated fbnA<sub>2A</sub>WT mice (Fig 7A, B). In fbnA<sub>2A</sub>KO mice CD11b activation was totally prevented in the SNc and Str (Fig 7A, B). Two-factors ANOVA revealed a significant effect of treatment, genotype and a treatment/genotype interaction (see Table 2 in A.I.).

**Discussion**

Antagonism of adenosine A<sub>2A</sub> receptors or their selective deletion in forebrain neurons produced similar protection of TH-positive nigral neurons in a multiple MPTP injections mouse model of PD. These complementary pharmacological and genetic means of A<sub>2A</sub> receptor disruption also attenuated the neurotoxin-triggered activations of astroglia and microglia along the nigrostriatal pathway.

Together these data provide evidence that therapeutically accessible A<sub>2A</sub> receptors located on forebrain neurons play a critical role in nigral dopaminergic neuron degeneration and inflammatory processes in the multiple MPTP injections mouse model of PD.

### **Pharmacological blockade of A<sub>2A</sub> receptors prevents MPTP-induced dopaminergic neuron degeneration and glial activation**

Systemic administration of the A<sub>2A</sub> receptor antagonist SCH58261 prevented the degeneration of nigrostriatal TH-positive neurons induced by repeated MPTP exposure in mice. Changes in number of TH-positive neurons correlated with changes in Nissl-stained (cresyl violet-positive) cells, indicating that MPTP treatment resulted in actual loss of dopaminergic neurons, which were rescued by SCH58261.

A neuroprotective effect of A<sub>2A</sub> receptor antagonists was previously observed upon acute administration of high MPTP doses in mice (Chen et al., 2001; Ikeda et al., 2002; Pierri et al., 2005; Yu et al., 2008). Here we report that neuroprotection with A<sub>2A</sub> antagonism can also be achieved upon multiple low-doses MPTP exposure. A number of studies have provided evidence that a repeated daily MPTP administration protocol similar to the one used here, presents histopathological features that more closely reproduce the human PD neuropathology, including apoptotic death of dopaminergic neurons (Jackson-Lewis et al., 1995; Tatton & Kish, 1997). Therefore, the present study further substantiates the neuroprotective potential of A<sub>2A</sub> antagonism in PD. To this regard, it is noteworthy that A<sub>2A</sub>R antagonists were shown to inhibit apoptotic neuronal death in hippocampal neurons (Silva et al., 2007).

Based on their differential location in the Str or other brain regions, A<sub>2A</sub> receptors may hold different levels of expression and intracellular signalling, reflecting A<sub>2A</sub> receptor multiple functions (Kull et al., 2000; Pedata et al., 2003; Rebola et al., 2005; Rosin et al., 2003; Shen et al., 2008). According to such

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varied roles of the A<sub>2A</sub> receptor, diverse effects have been attributed to A<sub>2A</sub> antagonists, ranging from symptomatic antiparkinsonian actions to neuroprotection in various neurodegenerative conditions (Alfinito et al., 2003; Blum et al., 2003; Chen et al., 1999; Melani et al., 2003; Monopoli et al., 1998; Popoli et al., 2002). Motor effects of A<sub>2A</sub> receptor antagonists are likely mediated by A<sub>2A</sub> receptors located on striatal neurons projecting to globus pallidus, whereas several mechanisms have been hypothesized for their neuroprotective effects, involving either neuronal or glial A<sub>2A</sub> receptors, though no single mechanism has yet been proven to prevail (Carta et al., 2003; Chen et al., 2001; Huang et al., 2006; Melani et al., 2003; Popoli et al., 1995; Pedata et al., 2003; Schwarzschild et al., 2006; Yu et al., 2008). Noticeably, in the present study neuroprotection by SCH58261 was achieved at doses similar to those effective in other neurodegenerative conditions, but several times lower than doses displaying a symptomatic efficacy in PD (Chen et al., 2001; Dall’Igna et al., 2003; Melani et al., 2003; Pinna et al., 2007), supporting the concept that different mechanisms might account for A<sub>2A</sub>-mediated neuroprotection or symptomatic effects.

SCH58261 fully prevented astroglia and microglia activation in the SNc, while only partially inhibiting astroglia and microglia reactivity in the striatum, in line with a partial protection of dopaminergic terminals. Noteworthy, A<sub>2A</sub> receptor antagonism prevented both astroglia and microglia activation in the SNc and Str at all time-points evaluated, in accordance with blockade of neurodegeneration.

Though the mechanism through which A<sub>2A</sub> receptor blockade produces neuroprotective effects in PD models is unclear, the modulation of neuroinflammation has been proposed as a likely target for neuroprotection (Hunot and Hirsch, 2003). Several findings have suggested that neuroinflammation may play an active role in the pathogenesis of neurodegeneration in PD, since focal inflammation has been described in the SNc of PD patients and MPTP-treated primates (Barcia et al., 2004; McGeer et al., 1988). Intriguingly, blockade of microglia reactivity in mice rescued dopamine neurons from acute MPTP toxicity (Wu et al., 2002). Moreover, in mice acutely treated with MPTP, dopamine neuron

neuroprotection by pre-treatment with an A<sub>2A</sub> antagonist was associated with an attenuation of astroglia and microglia activation in SNc and Str (Ikeda et al., 2002; Pierri et al., 2005), consistent with a causal relation between the two events.

**Selective deletion of neuron specific forebrain (fbn) A<sub>2A</sub> receptors prevents MPTP-induced dopamine neuron degeneration and glial activation.**

Previous studies evaluating A<sub>2A</sub> receptor-mediated neuroprotection have hypothesised several mechanisms that might underlie this process. In order to determine the role of neuronal *versus* glial A<sub>2A</sub> receptors in neuroprotection of dopamine neurons, we exploited genetically modified mice with selective depletion of A<sub>2A</sub> receptors from forebrain neurons (Bastia et al., 2005; Shen et al., 2008).

Importantly, in the fbnA<sub>2A</sub>KO mice, deletion of A<sub>2A</sub> receptor is not only spatially restricted (to forebrain A<sub>2A</sub> receptor) but is also temporally limited to postnatal A<sub>2A</sub> receptors (Bastia et al., 2005; Yu et al., 2008), thus avoiding potential confounds of compensatory responses to A<sub>2A</sub> receptor gene disruption during development as might occur in constitutive A<sub>2A</sub> knockout mice.

Our results revealed that selective deletion of A<sub>2A</sub> receptors from forebrain neurons totally prevented dopaminergic neuron loss in the SNc following multiple MPTP injections, while partially preventing damage to striatal dopaminergic terminals. A<sub>2A</sub>R deletion provided a greater protection of SNc neurons than the A<sub>2A</sub>R antagonist, as expected from a permanent as compared to a temporal pharmacological blockade of the receptor, in line with the reported half-life of SCH58261 of 2-3 h.

A<sub>2A</sub> receptors are located both pre- and post-synaptically in striatal and cortical neurons, and are expressed in microglial as well as astroglial cells (Cunha, 2001; Kust et al., 1999; Nishizaki et al., 2004; Rebola et al., 2005; Rosin et al., 2003). Positive modulation of postsynaptic signalling as well as of pre-synaptic release of neurotransmitters as glutamate and acetylcholine by A<sub>2A</sub> receptors have been described (Fredholm et al., 2003; Fuxe et al., 2003; Marchi et al., 2002; Popoli et al., 1995; Schiffmann et al., 2007; Schwarzschild et al., 2006). In addition, A<sub>2A</sub> receptors interfere with glia-mediated

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3 synthesis and release of neurotoxic factors such as COX-2, prostaglandins, nitric oxide and glutamate,  
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6 which have been hypothesised to play central roles in inflammatory processes and neuronal damage  
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8 (Fiebich et al., 1996; Li et al., 2001; Saura et al., 2005).  
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10 The present study, by showing that selective deletion of A<sub>2A</sub> receptors from forebrain neurons protects  
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12 dopaminergic neurons from MPTP toxicity, endorses a primary role of neuronal receptors in mediating  
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14 neuroprotection in this multiple injections MPTP model of PD. Since very low levels of A<sub>2A</sub> receptors  
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16 are expressed by dopaminergic neurons in the SNc, it is unlikely that a direct action at this level might  
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18 mediate neuroprotection from MPTP toxicity in the SNc. Rather, an indirect effect at the pre-synaptic  
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20 level, through an inhibition of A<sub>2A</sub>-mediated glutamate release, which contribute to neuronal damage,  
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22 could be envisaged (Aguirre et al., 2005; Battaglia et al., 2004; Monopoli et al., 1998; Popoli et al.,  
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24 2002). Interestingly, recent studies have reported a tight cross-talk between adenosine and GDNF  
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26 receptors, resulting in a fine modulation of glutamate and dopamine release (Gomes et al, 2006; 2009).  
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29 In the Str, A<sub>2A</sub>R blockade would impair GDNF-stimulated increase of corticostriatal glutamate release,  
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31 thus providing a beneficial effect on neurodegeneration. In addition, A<sub>2A</sub> receptor antagonism on  
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33 striatopallidal or subthalamic (STN) neurons might be protective from MPTP toxicity by modulating  
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35 excessive activation of subthalamic nucleus, thereby reducing excitotoxic glutamate efflux to SNc  
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37 neurons (Wallace et al., 2007).  
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40 A study by Yu and colleagues reported that fbnA<sub>2A</sub>KO mice from the same line as used here, were not  
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42 protected from striatal dopamine loss in response to acute MPTP exposure (in a single dose or multiple  
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44 doses over 4 hr; Yu et al., 2008). These results open to several compelling interpretations. First, the  
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46 cellular basis of A<sub>2A</sub> receptor-dependence of MPTP toxicity might vary depending on the duration of  
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48 toxin exposure, in line with the different type of neurotoxicity produced by acute as compared to  
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50 subchronic MPTP. Moreover, it should be taken into account the different parameters used to evaluate  
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52 nigrostriatal neurons damage. The drop of striatal dopamine levels assessed by Yu et al. may reflect  
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functional injury to dopaminergic terminals, whereas the measure of dopaminergic nigral neuron employed in our study may reflect an underlying neurodegenerative process in this area. All together results support the concept that A<sub>2A</sub> receptors display complex actions related to the duration of insult, cellular elements and brain areas targeted by neurodegenerative processes.

Lack of a glial reaction in fbnA<sub>2A</sub>KO mice, as compared to the robust astroglia and microglia activation in MPTP-treated control mice, indicates that deletion of neuronal A<sub>2A</sub> receptors may indirectly inhibit the inflammatory response. Glutamate is a main contributor to the complex neuron-glia cross-talk engaged by pathological events, which trigger both microglia and astroglia activation. For instance, by an action on NMDA receptors, glutamate release stimulates mitogen-activated protein kinases (MAPKs). Neuronal as well as glial p38 MAPK activation has been involved in cell suffering and apoptotic death, being activated and inducing several inflammatory mediators (Gianfriddo et al., 2004; Irving et al., 2000; Kawasaki et al., 1997; Piao et al., 2003). Therefore, A<sub>2A</sub> antagonists indirectly through a reduction of glutamate release might counteract glial reactivity and neuroinflammation in both SNc and Str (Melani et al., 2003, 2006). Activated glial cells, by the release of several toxic species as cytokines, free radicals, glutamate, is known to contribute to neuronal damage, and has been suggested to sustain a self-amplifying cycle which perpetuates MPTP toxicity (Hunot and Hirsh, 2003). Hence, interruption of such a detrimental vicious cycle might indirectly contribute to A<sub>2A</sub> receptor-dependent neuroprotection. Accordingly results suggest that in our model, operational glial A<sub>2A</sub> receptors did not contribute to MPTP-toxicity when A<sub>2A</sub> forebrain neuronal receptors were deleted. All together, our results suggest that A<sub>2A</sub> antagonists, by blocking neuronal A<sub>2A</sub> receptors, might act upstream in the cascade of toxic events and lead to an attenuation of dopamine neuron degeneration in PD.

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## Figures legend

**Fig 1: Adenosine A<sub>2A</sub> receptor antagonist SCH58261 prevents dopaminergic cell loss in the SNc.**

(A) shows representative sections immunostained for TH from SNc of mice sacrificed 3 days after MPTP treatment. Left insert shows TH-positive cells at higher magnification, right insert shows cresyl violet-stained sections; scale bar: 50 µm. Mice were treated with MPTP-HCl (20 mg/kg once a day for 4 days), plus SCH58261 (0.5 mg/kg) or vehicle (twice a day during MPTP treatment and once daily thereafter until sacrifice), and sacrificed 1, 3, 7 days after MPTP treatment. (B) shows analysis of TH immunostaining at 1, 3, 7 days after MPTP, reported as a percentage of TH-positive cells as compared to vehicle-treated mice. \* indicates p<0.001 versus vehicle; # indicates p<0.001 versus MPTP group, by Tukey's *post hoc* test.

**Fig 2: Adenosine A<sub>2A</sub> receptor antagonist SCH58261 attenuates degeneration of dopaminergic**

**terminals in the Str.** (A) representative sections immunostained for TH, from the Str of mice

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sacrificed 3 days after MPTP treatment. (B) Results from pharmacological blockade with SCH58261 or genetic A<sub>2A</sub>R depletion are presented. In the left column, - and + indicate the administration of vehicle or SCH58261 to MPTP-treated mice. In the right column + and – indicate fbnA<sub>2A</sub>WT and fbnA<sub>2A</sub>KO mice, respectively.

**Fig 3: Adenosine A<sub>2A</sub> receptor antagonist SCH58261 counteracts astroglia activation in the SNc and Str.** (A) shows representative sections immunostained for GFAP, from SNc (upper images) and Str (lower images) of mice sacrificed 3 days after MPTP treatment. Mice were treated as described in Fig 1. (B) shows analysis of GFAP immunostaining 1, 3, 7 days after MPTP, reported as percentage of GFAP-positive cells as compared to vehicle-treated mice in the SNc (left graph) and in the Str (right graph). \* indicates p<0.001 versus corresponding vehicle and MPTP+SCH58261 groups; # indicates p<0.001 versus corresponding MPTP group, by Tukey’s *post hoc* test.

**Fig 4: Adenosine A<sub>2A</sub> receptor antagonist SCH58261 counteracts microglia activation.** (A) Representative images from the SNc immunostained for CD11b as a marker of microglia activation. Mice were treated as described in Fig 1 and sacrificed 1, 3, 7 days after MPTP treatment. (B) CD11b analysis in SNc and Str was performed in grey-scale digitized images. The area occupied by grey values above a threshold was calculated and expressed as square pixels and as percentage of vehicle-treated mice. Tukey’s post hoc test: \* p<0.001 versus vehicle and MPTP+SCH58261 group; # p<0.001 versus MPTP group; ^, ^^ p<0.05, p<0.001 versus the indicated time point.

**Fig 5: fbnA<sub>2A</sub> KO mice are protected against MPTP-induced loss of dopaminergic cells in the SNc.** (A) shows representative sections from SNc immunostained for TH. Inserts show higher magnification of TH-labelled (left) and cresyl violet-labelled (right) cells. Mice were treated with MPTP (20 mg/kg once a day for 4 days) or vehicle. (B) shows analysis of TH immunostaining in

fbnA<sub>2A</sub>KO mice, reported as a percentage of TH-positive cells as compared to vehicle-treated mice.

Tukey's post hoc test: \* p<0.05 versus vehicle group; # p<0.05 versus WT MPTP group.

**Fig 6: Astroglia activation is attenuated in SNc and Str of fbnA<sub>2A</sub>KO mice.** (A) representative images from the SNc immunostained for GFAP, as a marker of astroglial cells. (B) Graphs show the analysis of GFAP immunostaining in SNc and Str, in fbnWT and fbnA<sub>2A</sub>KO mice treated with vehicle or MPTP. Tukey's post hoc test: \* p<0.001 versus vehicle group; # p<0.001 versus WT MPTP group.

**Fig 7: Microglia activation is prevented in SNc and Str of fbnA<sub>2A</sub>KO mice.** (A) representative images from the SNc immunostained for CD11b, as a marker of microglia activation. (B) Graphs show the analysis of CD11b immunostaining in SNc and Str, in fbnWT and fbnA<sub>2A</sub>KO mice treated with vehicle or MPTP. Tukey's post hoc test: \* p<0.001 versus vehicle group; # p<0.001 versus WT MPTP group.

**Table 1: unbiased evaluation of TH-IR neurons by stereological analysis in the SNc of mice following pharmacological blockade of A<sub>2A</sub>Rs with the antagonist SCH58261 or A<sub>2A</sub>R genetic depletion.** \* p<0.01 versus vehicle group. ^ p<0.01 versus the corresponding MPTP treated group.

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Tab 1. Stereological evaluation of TH-IR neurons in the substantia nigra pars compacta

Treatment	N	Density of TH-IR neurons/mm <sup>3</sup>
<i>pharmacological blockade of A<sub>2A</sub>R</i>		
Vehicle	3	29725.89 ± 439.56
MPTP	3	15211.79 ± 1300.06*
SCH + MPTP	3	23003.66 ± 495.76* ^
<i>fhnA<sub>2A</sub>R depletion</i>		
WT vehicle	3	27447.34 ± 1365.08
WT MPTP	3	14278.85 ± 2493.30*
KO vehicle	3	23654.36 ± 2277.74
KO MPTP	3	24267.96 ± 2277.83^

## TH immunoreactivity in SNc

Appendix A

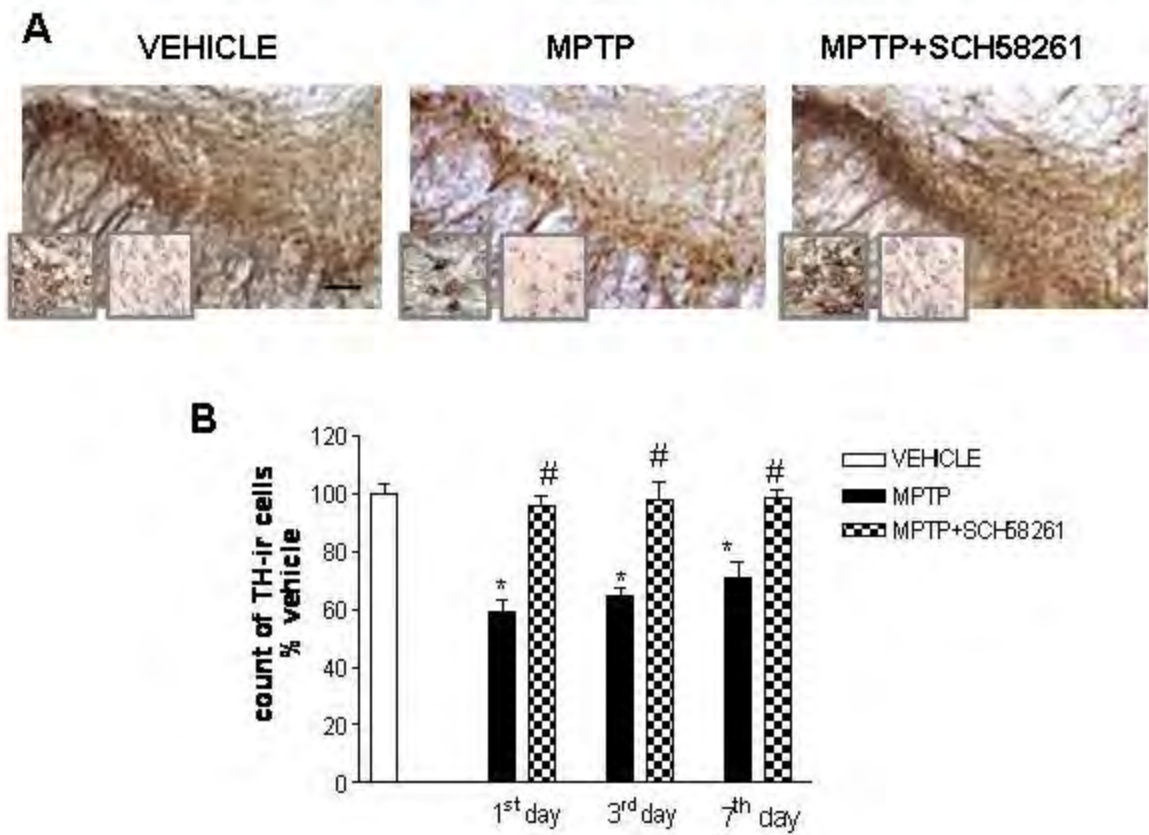


Fig 1

TH immunoreactivity in Str

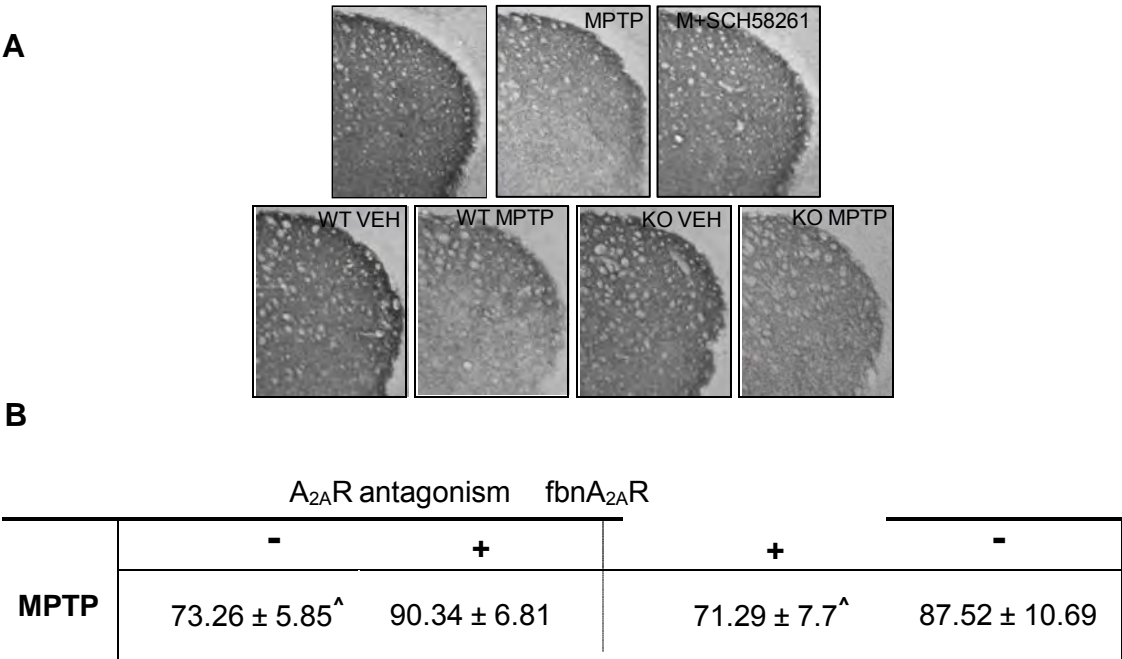
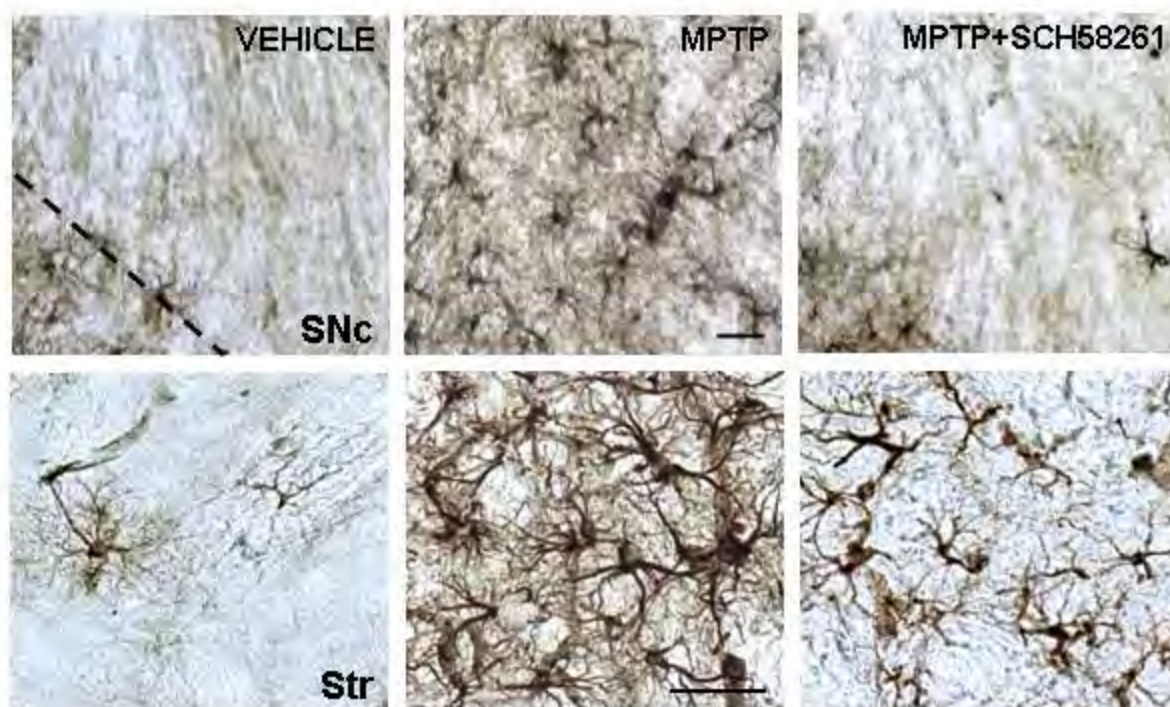


Fig 2



## GFAP immunoreactivity

A



B

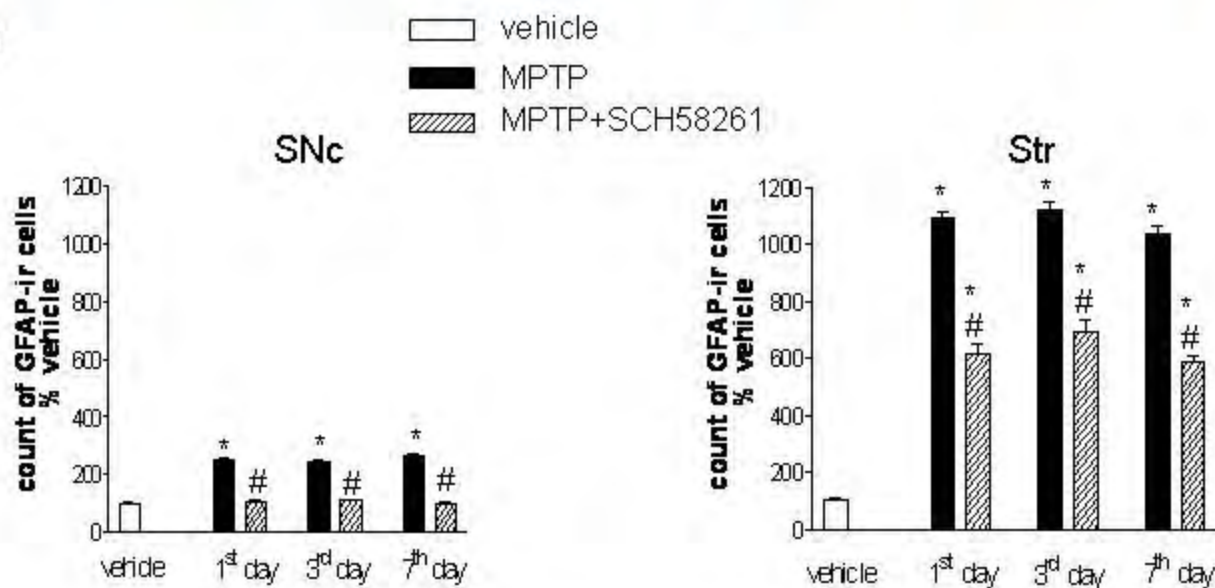


Fig.3

**A Substantia nigra**



**B**

**SNc**

**Str**

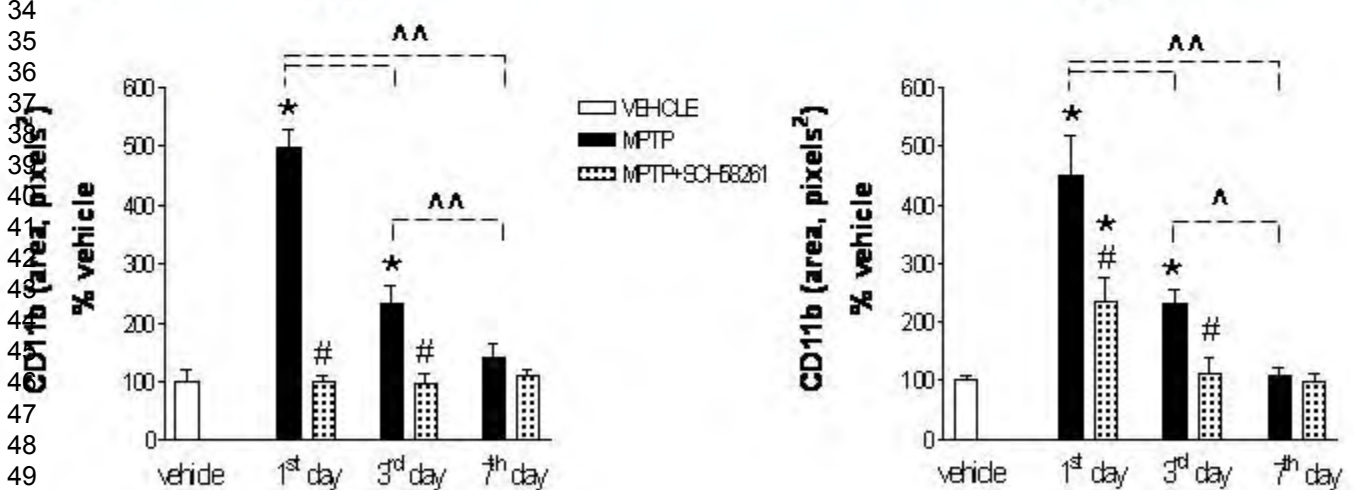


Fig 4



## TH immunoreactivity in SNc

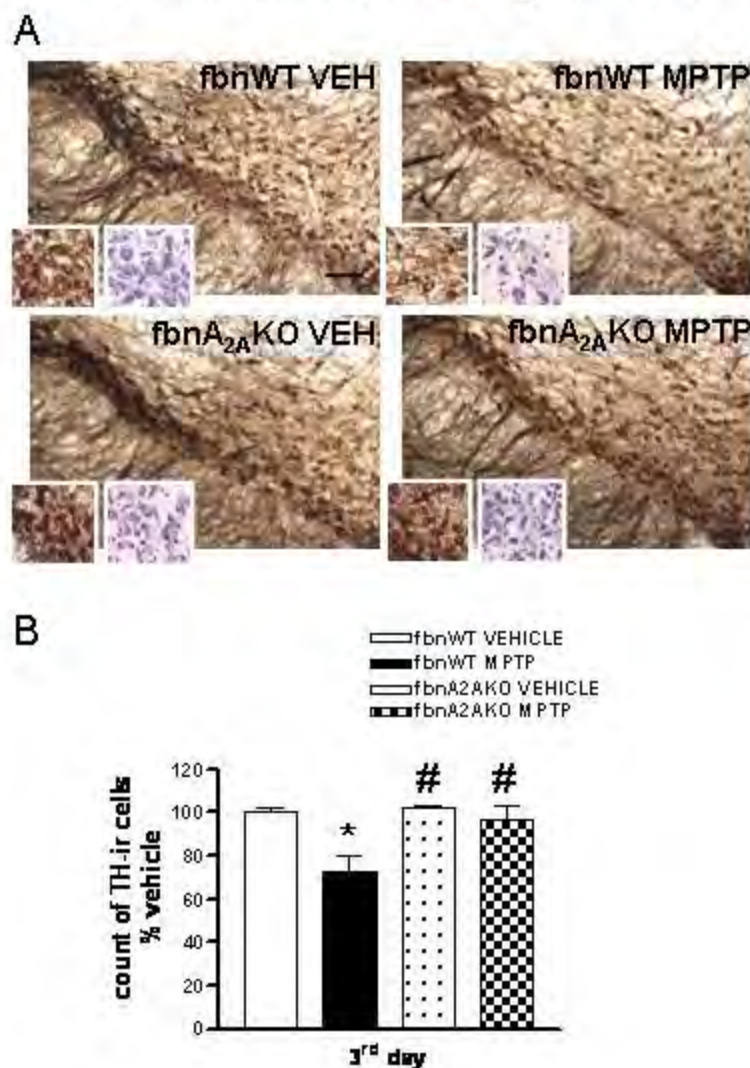


Fig 5

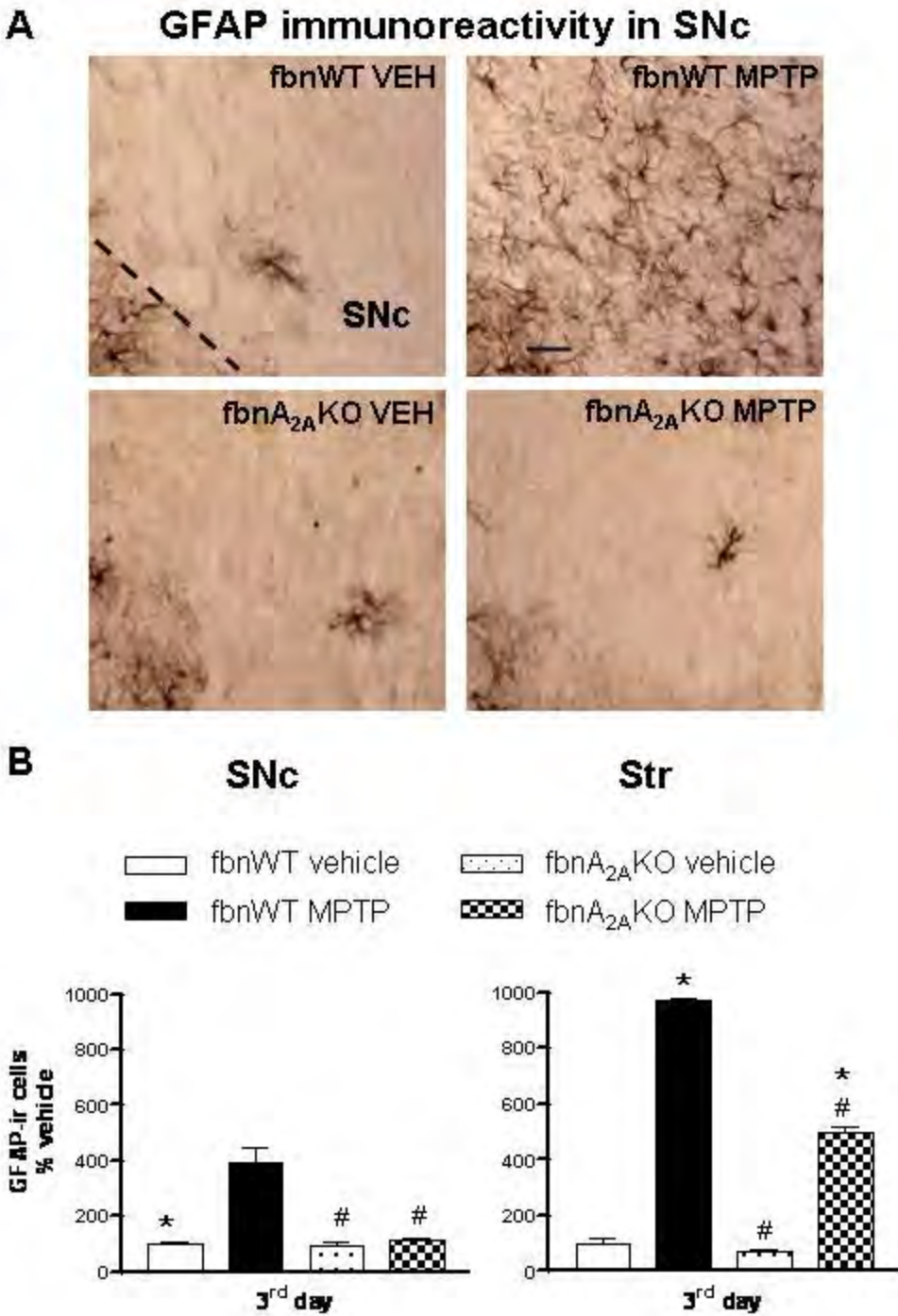
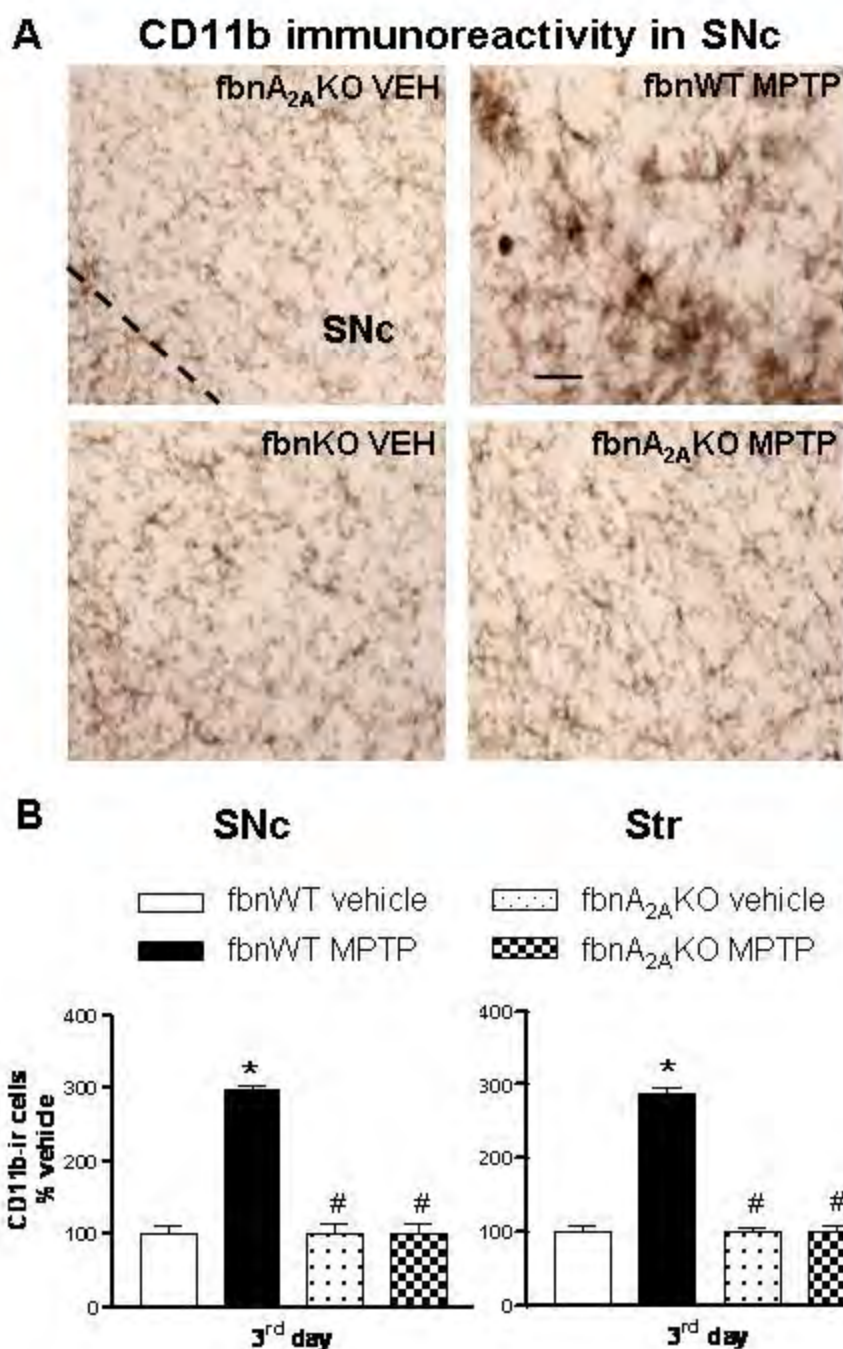


Fig 6



Additional Informations for online version only:

Table 2. F and P values from two factors ANOVA statistics

Experiment	Marker	Structure	Main effect of treatment		Main effect of time/genotype		Treatment-time/genotype interaction	
			<i>F</i> (2,47)	<i>P</i>	<i>F</i> (2,47)	<i>P</i>	<i>F</i> (4,47)	<i>P</i>
Pharmacological blockade of A <sub>2A</sub> R	TH	SNc	90.05	4.47E-18	1.53	n.s.	0.79	n.s.
	GFAP	SNc	826.99	0	0.67	n.s.	3.76	n.s.
		Str	1665.32	0	6.62	0.0025	2.17	n.s.
	CD11b	SNc	107.49	1.87E-18	35.12	4.01E-10	33.60	2.27E-13
		Str	41.01	1.73E-11	36.79	9.56E-11	15.38	2.06E-08
			<i>F</i> (1,26)	<i>P</i>	<i>F</i> (1,26)	<i>P</i>	<i>F</i> (1,26)	<i>P</i>
fbnA <sub>2A</sub> R depletion	TH	SNc	5.17	0.032	7.92	0.0092	3.35	n.s.
	GFAP	SNc	20.05	0.00013	23.29	5.32E-05	18.83	0.00019
		Str	248.94	7.85E-15	1634.94	5.29E-25	192.23	1.6E-13
	CD11b	SNc	14.14	0.0013	18.37	0.00040	33.00	1.55E-05
		Str	106.10	1.15E-09	111.57	7.37E-10	108.56	9.39E-10

Table 2 (A.I. for online version): F and P values from two-way ANOVA relative to experiments with A<sub>2A</sub> receptor antagonist (pharmacological blockade of A<sub>2A</sub>R), or in genetically manipulated mice (fbnA<sub>2A</sub>R depletion). n.s.: not significant.

**Generation of *fbnA<sub>2A</sub>KO* mice:** Postnatal forebrain neuron conditional A<sub>2A</sub>KO mice were generated using the Cre/*loxP* system based on the specificity of *CaMKII $\alpha$*  promoter. Near congenic (N6, C57Bl/6) male *A<sub>2A</sub><sup>flox/flox</sup>* mice (i.e., homozygous for the floxed allele of the *A<sub>2A</sub>R* gene) with or without a *CaMKII $\alpha$ -cre* transgene were used for this study. Tissue-specific postnatal recombination and disruption of the floxed *A<sub>2A</sub>R* alleles driven by *CaMKII $\alpha$ -cre* expression in forebrain neurons (i.e., *fbnA<sub>2A</sub>KO*) were confirmed by PCR and Western blot as described previously (Bastia et al, 2005, Yu et al, 2008).

**Immunohistochemistry:** For TH, GFAP and CD11b immunostaining, sections were incubated for 1 hr with 5% normal goat serum as blocking agent. The sections were then incubated overnight in primary antibodies (polyclonal rabbit anti-TH, 1:1000, Biomol, United Kingdom; monoclonal mouse anti-GFAP, 1:400, Sigma-Aldrich, Italy; monoclonal rat anti-mouse CD11b, 1:1000, Serotec, United Kingdom). Subsequently the sections were incubated in biotinylated secondary antibodies (goat anti-rabbit IgG for TH, goat anti-mouse IgG for GFAP and goat anti-rat IgG for CD11b, all purchased from Vector, United Kingdom). For visualization, the Avidin-peroxidase protocol (ABC, Vector) was applied, using 3,3'-diaminobenzidine (Sigma-Aldrich, Italy) as the chromogen. Sections were mounted into chrome-alum-gelatin-coated slides dehydrated and coverslipped.

**Stereological counting of TH-immunoreactive neurons:** TH-immunoreactive neurons were counted on both hemispheres. All stereological counting was performed using a Leica microscope (DMLB; Leica, Denmark) equipped with a camera (Basler Vision Technologies, Germany) and a stage connected to an *xyz* stepper (PRIOR ProScan) and the newCAST Visiopharm (Denmark) software. The SNc region was outlined at low magnification (5X) for area estimation. The number of TH-immunoreactive neurons was calculated under 63X magnification using randomized meander sampling

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and optical dissector methods. The cut thickness of sections was 50  $\mu\text{m}$  and the optical dissector height was 12  $\mu\text{m}$ . The top (13  $\mu\text{m}$ ) and bottom (25  $\mu\text{m}$ ) layers that shrunk during staining procedure were discarded. The sampling area covered 100% of the region of interest. The counting frame (8302,8  $\mu\text{m}^2$ ) applied the exclusion and inclusion lines and unbiased counting was performed by an experimenter blinded to the treatment. Results are presented as the mean of TH-immunoreactive neurons per  $\text{mm}^3 \pm$

SEM, calculated using the following formula:

**$D = 3Q / V \text{ sampling}$**

D = density of TH-ir cells per  $\text{mm}^3$

V sampling = area of the region of interest x dissector height

Q = total count of TH-ir neurons.



# Urate as a Predictor of the Rate of Clinical Decline in Parkinson Disease

ARCHIVES EXPRESS

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for the Parkinson Study Group DATATOP Investigators

**Background:** The risk of Parkinson disease (PD) and its rate of progression may decline with increasing concentration of blood urate, a major antioxidant.

**Objective:** To determine whether serum and cerebrospinal fluid concentrations of urate predict clinical progression in patients with PD.

**Design, Setting, and Participants:** Eight hundred subjects with early PD enrolled in the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) trial. The pretreatment urate concentration was measured in serum for 774 subjects and in cerebrospinal fluid for 713 subjects.

**Main Outcome Measures:** Treatment-, age-, and sex-adjusted hazard ratios (HRs) for clinical disability requiring levodopa therapy, the prespecified primary end point of the original DATATOP trial.

**Results:** The HR of progressing to the primary end point decreased with increasing serum urate concentrations (HR for highest vs lowest quintile=0.64; 95% confidence interval [CI], 0.44-0.94; HR for a 1-SD

increase=0.82; 95% CI, 0.73-0.93). In analyses stratified by *ct*-tocopherol treatment (2000 IU/d), a decrease in the HR for the primary end point was seen only among subjects not treated with *ct*-tocopherol (HR for a 1-SD increase=0.75; 95% CI, 0.62-0.89; vs HR for those treated=0.90; 95% CI, 0.75-1.08). Results were similar for the rate of change in the Unified Parkinson's Disease Rating Scale score. Cerebrospinal fluid urate concentration was also inversely related to both the primary end point (HR for highest vs lowest quintile=0.65; 95% CI, 0.44-0.96; HR for a 1-SD increase=0.89; 95% CI, 0.79-1.02) and the rate of change in the Unified Parkinson's Disease Rating Scale score. As with serum urate concentration, these associations were present only among subjects not treated with *ct*-tocopherol.

**Conclusions:** Higher serum and cerebrospinal fluid urate concentrations at baseline were associated with slower rates of clinical decline. The findings strengthen the link between urate concentration and PD and the rationale for considering central nervous system urate concentration elevation as a potential strategy to slow PD progression.

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**I**N HUMANS, URATE IS A MAJOR ANTIOXIDANT as well as the end product of purine metabolism.<sup>1,2</sup> Its high concentrations in cerebrospinal fluid (CSF) and blood have been attributed to a mutation in the urate oxidase gene occurring late in hominid evolution.<sup>3</sup> Oxidative damage is suspected to contribute to the neurodegenerative process in Parkinson disease (PD),<sup>4,5</sup> and antioxidants like urate may provide an endogenous defense against the development and progression of PD.

Prospective epidemiological studies have demonstrated that healthy individuals with higher blood urate concentrations are at reduced risk for developing PD.<sup>6-9</sup> Similarly, a lower risk of PD has also

been reported among individuals consuming diets that increase serum urate concentration<sup>10</sup> and among those with a history of gout.<sup>11,12</sup> Recently, we found that higher urate blood concentrations in patients in the early stages of PD predict a slower rate of disease progression, assessed by both clinical and neuroimaging measures.<sup>13</sup> These studies suggest that urate concentration measured systemically may serve as a robust predictor of the brain neurodegeneration that leads to the initiation and progression of PD.

The studies also raise the possibility that central nervous system urate directly protects against the neuronal degeneration underlying clinical deterioration in PD. Cerebrospinal fluid may more closely reflect

the microenvironment of degenerating neurons than does blood.<sup>14</sup> Accordingly, we used the clinical database from a completed multicenter, randomized, placebo-controlled trial (the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism [DATATOP] trial)<sup>15,16</sup> to test the hypothesis that higher urate concentrations in both CSF and blood specimens from patients with PD predict a slower rate of clinical disease progression.

## METHODS

### STUDY DESIGN

The DATATOP study was a 2-year, double-blind, randomized trial originally designed to test the hypothesis that long-term treatment of early PD with the monoamine oxidase type B inhibitor deprenyl (selegiline hydrochloride) and/or the antioxidant  $\alpha$ -tocopherol would extend the time until the emergence of disability requiring therapy with levodopa.<sup>15</sup> The 800 participants were enrolled between September 1987 and November 1988 at 28 sites across the United States and Canada.

### STUDY POPULATION

Subjects enrolled in the study had typical and early PD (Hoehn and Yahr stages 1 and 2) of less than 5 years' duration and were excluded if they used symptomatic PD medication or had severe tremor, serious dementia (Mini-Mental State Examination score <22), or depression (Hamilton Scale for Depression score  $\geq 16$ ). Subjects were reviewed and examined by neurologists who were PD specialists. After baseline evaluation, study participants were randomized according to a 2X2 factorial design to 1 of 4 treatment assignments: deprenyl (10 mg/d) and  $\alpha$ -tocopherol placebo,  $\alpha$ -tocopherol (2000 IU/d) and deprenyl placebo, active deprenyl and active  $\alpha$ -tocopherol, or double placebo.<sup>17</sup>

### SERUM AND CSF URATE CONCENTRATIONS AND COVARIATES

Urate concentration was measured in serum samples collected at the baseline visit prior to treatment assignment. Serum was shipped without freezing to a central commercial clinical laboratory (SciCor, Indianapolis, Indiana) for immediate enzymatic assay of urate concentrations, which were available for 774 of the 800 enrolled subjects. Values maintained in a digitized database were not analyzed with respect to disease progression outcome measures until their retrieval in May 2006 specifically for this purpose.

Cerebrospinal fluid was collected at baseline after overnight bed rest from 730 subjects (ie, 91.2% of enrollees, with technical difficulties in performing lumbar punctures precluding the collection from the others)<sup>18</sup> and at the end of the study in 486 subjects. Specimens were rapidly frozen for storage at  $-70^{\circ}\text{C}$  after first splitting all CSF collection tubes into aliquots with or without metabisulfite preservative added.<sup>18</sup> Baseline and final CSF urate concentrations were measured in 1991 by high-performance liquid chromatography with electrochemical detection from collection tubes containing the 18th to 20th milliliter of lumbar CSF flow in 2 selected subsets totaling 290 subjects who had provided both baseline and final CSF collections.<sup>19</sup> The values of CSF urate concentrations at baseline correlated well with those at the end of treatment or follow-up in both subsets (Spearman coefficient = 0.69;  $P < .001$ ), a result that supports the reproducibility of the assay as well as relatively stable within-person CSF urate concentrations. For the present

analyses, in 2008 we obtained CSF aliquots from the same collection tubes (containing no metabisulfite preservative) and repeated the measurement of urate concentrations by high-performance liquid chromatography with electrochemical detection. For these assays, 50  $\mu\text{M}$   $\alpha$ -methyl dopa served as an internal standard. Baseline CSF urate concentrations could be determined in 713 participants (ie, 97.7% of those from whom a baseline CSF sample was obtained and stored). Although mean CSF urate concentrations were lower than those measured in 1991, a good correlation was found between original urate concentrations and those measured in 2008 among the 277 individuals in both sets (Spearman coefficient = 0.72;  $P < .001$ ). Furthermore, baseline serum urate concentrations correlated more strongly with baseline CSF urate concentrations measured in 2008 ( $r = 0.73$ ) than in 1991 ( $r = 0.58$ ). These results provide evidence of the stability of urate in these samples and of the accuracy of CSF urate concentration measurements.

### CLINICAL EVALUATION AND OUTCOMES

Following the baseline visit and initiation of study drugs, subjects were scheduled for visits every 3 months until 24 months had elapsed.<sup>17</sup> At each visit the site investigator evaluated the subject for disability sufficient to require dopaminergic therapy, the primary end point for the study, and for the secondary response variables, including the Unified Parkinson's Disease Rating Scale (UPDRS) score (sum of the motor, cognitive, and activity of daily living subscale scores).<sup>17</sup> Because the UPDRS score is modified by the dopaminergic treatment instituted at the primary end point, the annualized rate of change in the UPDRS score was determined based on change from baseline to the primary end point (or the final visit if the primary end point was not reached) for each subject and was calculated as follows: [(total UPDRS score at the last assessment before initiation of dopaminergic treatment - total UPDRS score at baseline) / number of days between the 2 assessments]  $\times 365$  d/y. The vital status and date of death of participants in the DATATOP trial were collected in 2001 to 2002 as previously described.<sup>20</sup> The shortest time elapsed between enrollment and vital status update was 13 years. Information was available for 768 subjects with baseline serum urate concentration measurement.

### STATISTICAL ANALYSIS

In the original trial, the hazard ratios (HRs) for the primary end point were 0.50 (95% confidence interval [CI], 0.41-0.62) among patients assigned to deprenyl and 0.91 (95% CI, 0.74-1.12) among patients assigned to  $\alpha$ -tocopherol.<sup>17</sup> Accordingly, all of the analyses were adjusted for assignment to deprenyl vs placebo.

Cox proportional hazards models were used to estimate the HRs of reaching the primary end point according to quintiles of baseline serum urate concentration, adjusting for sex, age (in 5-year groups), and treatment assignment (deprenyl vs placebo). Initial analyses were conducted using quintiles based on the combined urate concentration distribution in men and women. However, because this categorization resulted in a markedly skewed distribution within sex as expected, we also conducted analyses based on sex-specific quintiles. Tests for trend were conducted by including serum urate concentration as a continuous variable in the proportional hazards models. Potential confounding was assessed by adjusting the regression analyses for body mass index (BMI) (calculated as weight in kilograms divided by height in meters squared) and use of antihypertensive drugs or nonsteroidal anti-inflammatory drugs (use vs no use). With the exception of BMI, these adjustments did not affect the results. Therefore, only the treatment-, age-, and sex-adjusted results or the treatment-, age-, sex-, and BMI-

**Table 1. Baseline Characteristics of Study Participants According to Quintiles of Baseline Serum Urate Concentration**

Characteristic	Baseline Serum Urate Concentration Quintile					All Subjects
	1	2	3	4	5	
Serum urate concentration, mg/dL	<3.90	3.91-4.60	4.61-5.20	5.21-6.20	2:6.21	5.1 <sup>a</sup>
Subjects, No.	162	140	149	165	158	774
Female, %	75.9	40.7	24.2	17.0	12.7	34.1
Age, median, y	62	63	61	63	63	62
BMI, mean	23.7	26.2	26.1	27.1	28.4	26.3
Current smokers, %	9.3	5.7	12.1	10.3	6.3	8.8
Using antihypertensive drugs for hypertension, %	6.8	7.1	7.4	8.5	15.7	9.2
Using thiazides, %	2.5	2.9	3.4	4.2	6.9	4.0
Using NSAIDs, %	22.2	22.1	20.1	27.3	17.6	21.9
Cardiac comorbidity, %	17.9	25.0	24.8	27.9	36.5	26.5
Time since onset per rater, y	1.9	2.0	2.0	2.0	1.9	2.0
Total UPDRS score, mean	25.1	25.7	25.4	26.4	24.5	25.5
UPDRS rest tremor score, mean	4.5	4.8	4.4	4.7	4.6	4.6
MMSE score, mean	28.8	28.9	29.0	28.8	28.8	28.9

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); MMSE, Mini-Mental State Examination; NSAIDs, nonsteroidal anti-inflammatory drugs; UPDRS, Unified Parkinson's Disease Rating Scale.

<sup>a</sup>Value is expressed as the mean.

adjusted results are presented. Possible interactions were explored by including the cross-product of serum urate concentration (continuous variable) with age (continuous in years), sex, or deprenyl and ct-tocopherol treatments in the proportional hazards model. No interaction terms were significant, and only results that do not include these terms are reported. The results of these exploratory analyses, however, suggested a possible interaction between ct-tocopherol treatment and serum urate concentration. Because both ct-tocopherol and urate have antioxidant properties, this interaction has some biological plausibility. This interaction was examined further by estimating the HRs for the primary outcome in groups of subjects classified according to both their serum urate concentration and treatment group. The relationship between serum urate concentration and rate of change in the UPDRS score (between baseline and the last visit before reaching the primary end point) was assessed by linear regression with adjustment for treatment, age, and sex using both common quintiles of serum urate concentration and sex-specific quintiles. This analysis was complemented by a repeated-measures analysis using all available UPDRS score determinations. This analysis was conducted by fitting a linear mixed model with random intercept and slope and fixed effects for treatment, age, sex, urate concentration, and the interaction between urate concentration and ct-tocopherol treatment. The association between serum urate concentration and time from study enrollment until death was investigated using Cox proportional hazards models adjusted for treatment, age, sex, and smoking history (pack-years), with or without further adjustment for cardiac morbidity at baseline. Analyses for CSF urate concentration were conducted in the same manner.

## RESULTS

### SERUM URATE CONCENTRATION

Serum urate concentration at baseline was available for 774 of the 800 subjects (96.8%; 510 men and 264 women) enrolled in the trial. Selected characteristics of these subjects are shown in **Table 1**. As expected, serum urate concentrations correlated positively with being male, BMI, use of thiazide diuretics, and hypertension. Use of cal-

cium channel blockers was reported by only 17 patients and showed no relationship to serum urate concentration.

Overall, 369 (47.7%) of these participants progressed to disability sufficient to require levodopa therapy during follow-up. The HR of reaching this primary end point declined with increasing concentrations of serum urate ( $P$  for trend = .002) and was 36% lower among subjects in the top quintile as compared with those in the bottom quintile of serum urate concentration (HR = 0.64; 95% CI, 0.44-0.94) (**Table 2**). This association was stronger in men than in women, although a test for interaction of urate concentration with sex was not significant ( $P = .54$ ). Further, in both sexes, the HR for reaching the primary end point decreased with increasing BMI ( $P$  for trend = .05 in men,  $P$  for trend = .02 in women). After adjustment for BMI, the association between serum urate concentration and the primary clinical end point was partially attenuated; the HRs for a 1-SD increase in serum urate concentration were 0.89 in all subjects ( $P = .07$ ), 0.85 in men ( $P = .04$ ), and 1.01 in women ( $P = .94$ ).

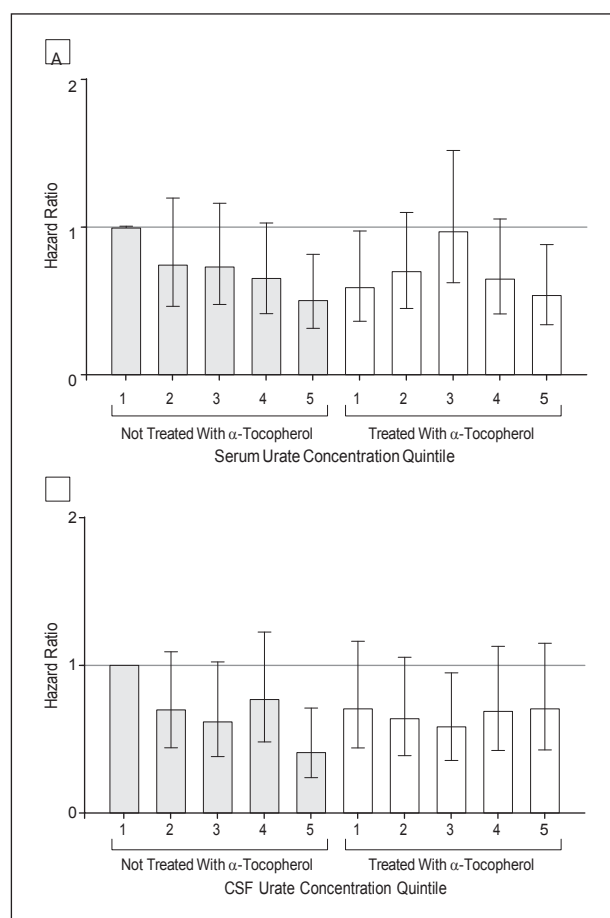
When subjects were classified simultaneously according to serum urate concentration and ct-tocopherol treatment, a decreasing HR for reaching the primary end point with increasing serum urate concentration was observed among untreated subjects (HR = 0.75; 95% CI, 0.62-0.89;  $P = .001$ ) but not among those treated (HR = 0.90; 95% CI, 0.75-1.08;  $P = .24$ ), consistent with comparisons of baseline urate concentration quintiles (**Figure 1A**) and unadjusted Kaplan-Meier analyses (eFigure; <http://www.archneurol.com>) in subgroups without or with ct-tocopherol treatment. Conversely, randomization to ct-tocopherol treatment appeared to lower the HR of reaching the primary end point among subjects in the lowest quintile of serum urate concentration (HR = 0.59; 95% CI, 0.36-0.97) but not among those with a higher serum urate concentration (**Figure 1A**). Further analyses were conducted within sex. In men, the HRs for a 1-SD increase in serum urate concentration were 0.74 (95% CI, 0.59-0.92;  $P = .008$ ) among subjects not receiving ct-tocopherol and



**Table 2. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Serum Urate Concentration or Corresponding to a 1-SD Increase in Serum Urate Concentration<sup>a</sup>**

Serum Urate Concentration Quintile	Serum Urate Concentration, mg/dL	All Subjects (n=774)			Men (n=510)			Women (n=264)		
		No.	HR (95% CI)	P Value	No.	HR (95% CI)	P Value	No.	HR (95% CI)	P Value
1	<3.90	162	1 [Reference]		39	1 [Reference]		123	1 [Reference]	
2	3.91-4.60	140	0.88 (0.62-1.25)	.47	83	0.88 (0.52-1.49)	.63	57	0.90 (0.53-1.51)	.68
3	4.61-5.20	149	1.04 (0.73-1.47)	.83	113	1.17 (0.71-1.91)	.54	36	0.86 (0.45-1.62)	.63
4	5.21-6.20	165	0.80 (0.55-1.15)	.23	137	0.78 (0.48-1.29)	.33	28	1.34 (0.68-2.64)	.40
5	≥6.21	158	0.64 (0.44-0.94)	.02	138	0.67 (0.41-1.11)	.12	20	0.58 (0.23-1.50)	.26
1-SD increase in serum urate concentration			0.82 (0.73-0.93)	.002		0.81 (0.70-0.94)	.005		0.89 (0.71-1.12)	.32

Abbreviations: CI, confidence interval; HR, hazard ratio.

<sup>a</sup>A 1-SD increase indicates an increase of 1.4 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).**Figure 1.** Hazard ratio for reaching the primary end point according to assignment to ct-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration (referenced to placebo-treated subjects in the lowest quintile). Error bars indicate 95% confidence intervals for hazard ratios adjusted for age, sex, and treatment group (deprenyl or placebo).

0.88 (95% CI, 0.71-1.08;  $P=.21$ ) among subjects receiving ct-tocopherol. In women, the corresponding HRs were 0.73 (95% CI, 0.52-1.02;  $P=.06$ ) for subjects not receiving ct-tocopherol and 1.04 (95% CI, 0.69-1.59;  $P=.84$ ) for subjects receiving ct-tocopherol. The interaction between ct-tocopherol and serum urate concentration was nonsignificant for men and for women ( $P$  for interaction=.55 in men, and  $P$  for interaction=.06 in women).

No significant interaction was found between serum urate concentration and deprenyl treatment; a decreasing HR with increasing serum urate concentration was found in the placebo-placebo and deprenyl-placebo groups but not in the placebo-ct-tocopherol and deprenyl-ct-tocopherol groups (eTable 1).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 760 of the 774 subjects with baseline serum urate concentrations. Overall, the rate of UPDRS score change declined with increasing serum urate concentration ( $P$  for trend=.03). As observed previously for the primary end point, results were more robust in men, although there was no statistically significant interaction with sex. Among men, the adjusted rate of UPDRS score change declined from 14.8 points per year for subjects in the lowest quintile of serum urate concentration to 8.9 points per year for those in the highest quintile ( $P$  for trend=.03); comparable results among women were 11.0 and 8.2 points per year, respectively ( $P$  for trend=.35). The relationship between serum urate concentration and the rate of UPDRS score change was modified by ct-tocopherol treatment ( $P$  for interaction=.009) (Figure 2A). In separate models, among subjects not assigned to receive ct-tocopherol, the rate of UPDRS score change was 9.8 points per year lower in the highest serum urate concentration quintile than in the lowest quintile ( $P=.003$ ), whereas no difference was observed for subjects assigned to receive ct-tocopherol (0.5 points per year higher in the highest quintile as compared with the lowest quintile;  $P=.89$ ). In analyses based on repeated measures, the overall association between higher urate levels at baseline and a slower rate of UPDRS score increase was even stronger ( $P=.001$ ). There was also a significant interaction between urate concentration and ct-tocopherol treatment ( $P=.003$ ), and consistent with results observed in the primary analyses, higher levels of serum urate were strongly associated with a slower rate of UPDRS score increase among patients not treated with ct-tocopherol ( $P=.001$ ) but not in those treated with ct-tocopherol ( $P=.37$ ). No significant interaction was found between serum urate concentration and deprenyl treatment (eTable 2).

Two hundred eleven men (41.4%) and 81 women (30.7%) were identified as having died after 13 years of

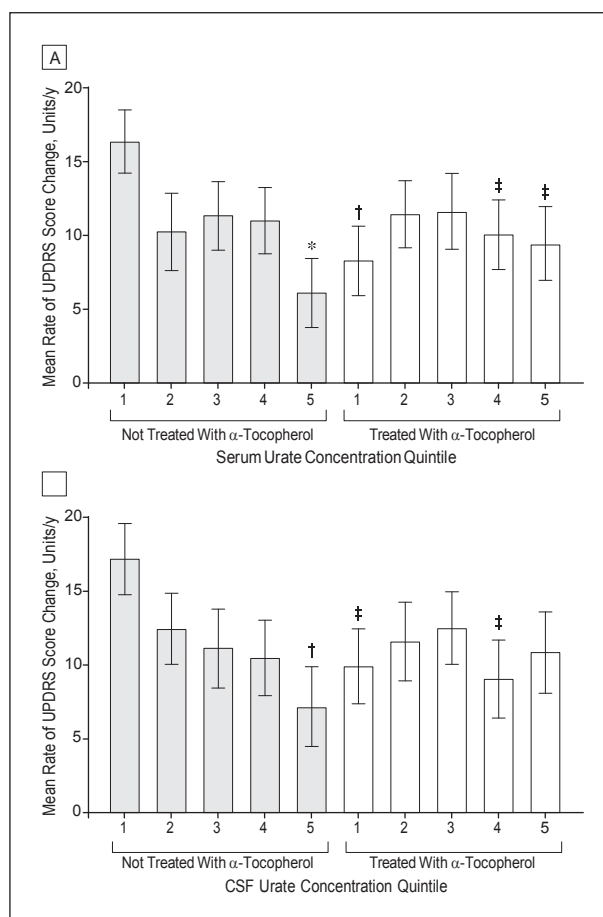
follow-up. In men and women combined, after adjustment for deprenyl treatment, age, sex, and pack-years of smoking (Table 3) and after adjustment for deprenyl treatment, age, sex, pack-years of smoking, and cardiac comorbidity at baseline (Table 4), serum urate concentration was not significantly associated with mortality. In men, however, the relationship between serum urate concentration and mortality was a U-shaped curve, with the lowest mortality in the fourth quintile of urate concentration. In women, a suggestion of increased mortality at any urate concentration higher than that in the lowest quintile was not substantiated statistically. No significant interactions between serum urate concentration and *ct*-tocopherol were found in analyses on mortality.

### CSF URATE CONCENTRATION

Mean urate concentrations in CSF collected at baseline were higher in men (0.42 mg/dL) than in women (0.28 mg/dL) and, as expected, were substantially lower than in serum.<sup>21</sup> Despite the lower concentrations of CSF urate, a strong correlation was found between CSF and serum urate concentrations ( $r=0.73$ ;  $P<.001$ ).

The primary clinical end point of disability was reached by 342 of the 713 subjects (48%) for whom CSF urate concentrations were available. Overall, the HR of reaching the primary end point of disability was significantly lower among individuals with higher concentrations of CSF urate. The HR comparing subjects in the highest quintile of CSF urate concentration with those in the lowest quintile was 1.65 (95% CI, 0.44-0.96;  $P=.03$ ); the HR associated with a 1-SD increase in CSF urate concentration was 0.89 (95% CI, 0.79-1.02;  $P=.09$ ) (Table 5). Results were not significantly different by sex, although a strong interaction was found between *ct*-tocopherol assignment and CSF urate concentration ( $P$  for interaction=.009) (Figure 1B). As for serum urate concentration, a significant decrease in the HRs for the primary end point with increasing CSF urate concentration was observed only among subjects not receiving *ct*-tocopherol. The HR corresponding to a 1-SD increase in CSF urate concentration was 0.77 (95% CI, 0.62-0.96;  $P=.02$ ) among men not treated with *ct*-tocopherol and 1.10 (95% CI, 0.90-1.34;  $P=.34$ ) among those receiving *ct*-tocopherol. In women, the corresponding HRs were 0.64 (95% CI, 0.40-1.03;  $P=.07$ ) for subjects not assigned to *ct*-tocopherol and 0.77 (95% CI, 0.43-1.37;  $P=.37$ ) for subjects treated with *ct*-tocopherol. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 3).

The change in UPDRS score between baseline and either the time of reaching the primary end point or the end of follow-up was available for 702 of the 713 subjects with baseline CSF urate concentrations. Overall, the rate of UPDRS score change was not related significantly to CSF urate concentration. As observed for serum urate concentration, however, the relationship between CSF urate concentration and the rate of UPDRS score change was modified by *ct*-tocopherol treatment ( $P$  for interaction=.04) (Figure 2B). Among subjects not treated with *ct*-tocopherol, the rate of UPDRS score change declined with increasing CSF urate concentrations ( $P$  for trend=.05). Con-



**Figure 2.** Mean annualized rate of Unified Parkinson's Disease Rating Scale (UPDRS) score change according to assignment to *ct*-tocopherol (vitamin E) and quintile of baseline serum (A) or cerebrospinal fluid (CSF) (B) urate concentration. Error bars indicate standard errors of the mean adjusted for age, sex, and treatment group (deprenyl or placebo). \*Significantly different from the placebo-treated subjects in the lowest quintile ( $P<.001$ ). †Significantly different from the placebo-treated subjects in the lowest quintile ( $P<.01$ ). ‡Significantly different from the placebo-treated subjects in the lowest quintile ( $P<.05$ ).

versely, randomization to *ct*-tocopherol treatment appeared to lower the rate of UPDRS score change among subjects in the lowest quintile of urate concentration measured either in CSF (Figure 2B) or in serum (Figure 2A) but not among those with higher urate concentrations. No significant interaction was found between CSF urate concentration and deprenyl treatment (eTable 4).

### COMMENT

Among subjects with early PD participating in a large randomized trial, we found that both serum and CSF urate concentrations measured at baseline were inversely related to clinical progression of PD. The internal consistency of the results across the primary and secondary end points supports their validity. These findings, like data from a similar early PD trial (the Parkinson Research Examination of CEP-1347 Trial [PRECEPT] study),<sup>13</sup> demonstrate a robust link between blood urate concentrations and the rate of clinical progression in PD. In addition, the association of CSF urate concentration with disease progression

**Table 3. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), and Pack-Years of Smoking**

Serum Urate Concentration Quintile	All Subjects (n=768)		Men (n=504)		Women (n=264)	
	HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
1	1 [Reference]		1 [Reference]		1 [Reference]	
2	1.17 (0.77-1.78)	.47	0.66 (0.38-1.15)	.14	1.68 (0.90-3.11)	.10
3	1.20 (0.78-1.83)	.41	0.66 (0.38-1.12)	.12	1.30 (0.64-2.66)	.47
4	1.11 (0.72-1.72)	.62	0.60 (0.35-1.02)	.06	1.88 (0.89-3.97)	.10
5	1.48 (0.96-2.27)	.06	0.89 (0.53-1.50)	.67	1.96 (0.89-4.33)	.10

Abbreviations: CI, confidence interval; HR, hazard ratio.

**Table 4. Hazard Ratios for Death From Any Cause According to Common Quintiles of Baseline Serum Urate Concentration, Adjusted for Age, Sex, Treatment Group (Deprenyl or Placebo), Pack-Years of Smoking, and Cardiac Comorbidity at Baseline**

Serum Urate Concentration Quintile	All Subjects (n=768)		Men (n=504)		Women (n=264)	
	HR (95% CI)	P Value	HR (95% CI)	P Value	HR (95% CI)	P Value
1	1 [Reference]		1 [Reference]		1 [Reference]	
2	1.12 (0.73-1.71)	.61	0.63 (0.36-1.10)	.10	1.66 (0.90-3.08)	.11
3	1.14 (0.74-1.74)	.56	0.63 (0.37-1.08)	.09	1.29 (0.63-2.64)	.49
4	1.05 (0.68-1.62)	.83	0.56 (0.33-0.95)	.03	1.85 (0.87-3.93)	.11
5	1.38 (0.89-2.12)	.15	0.83 (0.49-1.39)	.47	1.89 (0.83-4.30)	.13

Abbreviations: CI, confidence interval; HR, hazard ratio.

**Table 5. Hazard Ratios for Reaching the Primary End Point According to Common Quintiles of Baseline Cerebrospinal Fluid Urate Concentration or Corresponding to a 1-SD Increase in Cerebrospinal Fluid Urate Concentration<sup>a</sup>**

CSF Urate Concentration Quintile	CSF Urate Concentration, mg/dL	All Subjects			Men			Women		
		No.	HR (95% CI)	P Value	No.	HR (95% CI)	P Value	No.	HR (95% CI)	P Value
1	<0.23	143	1 [Reference]		38	1 [Reference]		105	1 [Reference]	
2	0.24-0.32	143	0.78 (0.55-1.10)	.16	81	1.05 (0.61-1.81)	.85	62	0.65 (0.38-1.11)	.11
3	0.32-0.39	144	0.70 (0.48-1.01)	.06	109	0.96 (0.57-1.63)	.89	35	0.51 (0.26-1.03)	.06
4	0.39-0.50	142	0.84 (0.58-1.22)	.36	117	1.08 (0.64-1.80)	.78	25	0.72 (0.34-1.55)	.40
5	2:0.51	141	0.65 (0.44-0.96)	.03	128	0.85 (0.51-1.43)	.54	13	0.47 (0.16-1.39)	.17
1-SD increase in CSF urate concentration			0.89 (0.79-1.02)	.09		0.93 (0.80-1.07)	.28		0.79 (0.57-1.10)	.17

Abbreviations: CI, confidence interval; CSF, cerebrospinal fluid; HR, hazard ratio.

<sup>a</sup>A 1-SD increase indicates an increase of 0.16 mg/dL. The HRs are adjusted for age, sex, and treatment group (deprenyl or placebo).

strengthens the possibility that brain urate concentration (or its determinants) might protect against the neurodegeneration of PD. Taken together, these data establish urate as the first molecular predictor of clinical progression in PD and provide a rationale for investigating the possibility that a therapeutic increase of urate in patients with PD might act favorably to slow the disease course. Interestingly, the inverse relationship between urate concentration and clinical progression was not observed among patients randomized to  $\alpha$ -tocopherol at a dosage of 2000 IU/d, suggesting that there may be an interaction between these antioxidants.

There is strong evidence that oxidative stress and nitrative stress are major pathogenetic mechanisms in PD.<sup>13,22,23</sup> Urate is an effective antioxidant,<sup>1</sup> peroxynitrite scavenger,<sup>24-27</sup> iron chelator,<sup>28</sup> and ascorbate stabilizer.<sup>29</sup> In cellu-

lar models of PD neurodegeneration, urate can reduce oxidative stress, mitochondrial dysfunction, and cell death occurring spontaneously in culture or induced by the pesticide rotenone, 1-methyl-4-phenylpyridinium, glutamate, and iron ions.<sup>30-32</sup> Although urate appears to have the potential for neuroprotection, it is possible that the predictive association between urate concentration and PD progression reflects instead the effect of a urate precursor, such as adenosine or inosine, or another determinant of systemic and CSF urate concentrations.

As compared with serum urate concentration, the weaker association of CSF urate concentration to clinical progression of PD may seem at odds with the hypothesis that urate (or its metabolic precursors) exerts a beneficial effect through presence in the central nervous system. The CSF urate concentrations, how-



ever, display a strong caudorostral gradient from the lumbar space, with lumbar region values approximately 50% higher than those arising at the cisterna magna (brainstem) level.<sup>33,34</sup> Although we consistently used CSF aliquots obtained from the 18th to 20th milliliter of CSF flow, variations in CSF circulation patterns between patients<sup>35</sup>—along with freezer storage for 20 years—may have contributed to a reduction of the accuracy of this measure compared with assays of freshly collected serum samples. In addition to technical variability, substantial biological differences between the urate in CSF sampled from the subarachnoid space and that in the degenerating neurons themselves may lessen the strength of a CSF urate concentration–clinical progression correlation in PD.

The finding that the inverse relationship between urate concentration and clinical progression of PD was modified by *ct*-tocopherol treatment was unforeseen because, as originally reported, no favorable effect of *ct*-tocopherol on PD progression was found among study participants in the DATATOP trial.<sup>16</sup> The mechanisms for a possible interaction between urate and *ct*-tocopherol remain uncertain. Although hydrophilic (eg, urate) and hydrophobic (eg, *ct*-tocopherol) antioxidants target different subcellular compartments, their functional interactions have been described.<sup>36,37</sup> Further, *ct*-tocopherol at doses commonly used in vitamin supplements may reduce concentrations of other endogenous antioxidants,<sup>38,39</sup> and *ct*-tocopherol at high doses may have pro-oxidant rather than antioxidant effects.<sup>40,41</sup> Alternatively, a simple competitive interaction or “ceiling effect” may have contributed to the observed lack of *ct*-tocopherol benefits among patients with PD with higher urate concentrations as well as to the loss of the inverse association between urate concentration and PD progression among those receiving supplemental *ct*-tocopherol. Regardless of the mechanism for a possible interaction between *ct*-tocopherol and urate, our results raise the possibility that such an interaction may have obscured a protective effect of *ct*-tocopherol among those subjects with low baseline concentrations of urate in the DATATOP trial. Further investigations are therefore needed to consider the possibility that *ct*-tocopherol supplementation may be beneficial in individuals with low urate concentrations.

Serum urate may also affect the progression of cognitive impairment in that higher concentrations seem to be associated with slower rates of cognitive decline and lower risk of dementia.<sup>42–44</sup> As in the present study, among participants in a randomized trial, this association was observed in patients treated with placebo but not in those treated with *ct*-tocopherol.<sup>43</sup> Higher serum urate concentration has also been linked to a lower rate of worsening in Huntington disease.<sup>45</sup> Although each of these neurodegenerative disorders manifests differently from PD, the relationships between urate concentration and these disorders may be indicative of a more general influence of urate (or its precursors) on neuronal cell death.

The main results of this study are strikingly consistent with those recently reported from the PRECEPT study.<sup>13</sup> Although the overall inverse relationship between serum urate concentration and the clinical pro-

gression of PD was greater in the PRECEPT study than in the DATATOP study, results among subjects in the DATATOP study not assigned to *ct*-tocopherol were virtually identical to those observed in the PRECEPT study (which did not include an *ct*-tocopherol treatment arm). In both trials, HRs for risk of disability progression showed a decline in patients whose values were higher than the median concentration but still within the normal range of serum urate concentrations. Moreover, in both trials, the concentration-dependent inverse relationship was robust in men but weak and nonsignificant among women. This consistent difference between men and women could result in part from a biological effect of sex on urate mechanisms in PD<sup>46</sup> or could simply reflect the small number of women with urate concentrations high enough to slow disease progression if urate were protective.

A potentially therapeutic effect of elevating serum urate concentration warrants consideration. Urate levels can be elevated by dietary means, including increased intake of fructose<sup>47–49</sup> or purines,<sup>50</sup> or by pharmacological means. The latter may include administration of the purine metabolite and urate precursor inosine, which is being investigated as a therapy for multiple sclerosis.<sup>26,27</sup> The potential benefit of elevating urate concentration in individuals with PD, however, has to be weighed against possible adverse effects, which may include an increased risk of hypertension, coronary heart disease, and stroke<sup>6,51–53</sup> in addition to the known risks of gout and urolithiasis. Available data are therefore insufficient to support a therapeutic recommendation.

The discovery of a urate link to PD progression was achieved through additional analyses of 2 rigorously conducted clinical trials whose databases were made available to test an unforeseen hypothesis months<sup>54</sup> or decades<sup>18,19</sup> after conclusion of the primary investigations. These latent insights highlight a broader opportunity to achieve further advances through explorations of the growing repository of high-quality data collected from neuroprotection trials of PD and other neurodegenerative disorders.

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**Author Contributions:** Dr Schwarzschild had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Ascherio, Kieburtz, Shoulson, and Schwarzschild. *Acquisition of data:* LeWitt, Xu, Matson, Marras, Rudolph, Schwid, Tennis, Tanner, Beal, Lang, Fahn, Shoulson, and Schwarzschild. *Analysis and interpretation of data:* Ascherio, LeWitt, Xu, Eberly, Watts, Marras, Kieburtz, Bogdanov, Tennis, Tanner, Lang, Oakes, Fahn, Shoulson, and Schwarzschild. *Drafting of the manuscript:* LeWitt, Oakes, and Schwarzschild. *Critical revision of the manuscript for important intellectual content:* Ascherio, LeWitt, Xu, Eberly, Watts, Matson, Marras, Kieburtz, Rudolph, Bogdanov, Tennis, Tanner, Beal, Lang, Oakes, Fahn, Shoulson, and Schwarzschild. *Statistical analysis:* Eberly and Oakes. *Obtained funding:* Ascherio, LeWitt, Shoulson, and Schwarzschild. *Administrative, technical, and material support:* LeWitt, Xu, Watts, Matson, Kieburtz, Rudolph, Schwid, Fahn, Shoulson, and Schwarzschild. *Study supervision:* Beal and Schwarzschild.

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**Abstract:** Lewy bodies are found in Parkinson disease and related disorders, and are extensively phosphorylated at Ser-129. Recent studies suggest that S129 phosphorylation may mediate  $\alpha$ -synuclein aggregation and neurotoxicity, but have had conflicting results. We used recombinant adeno-associated virus (rAAV) to overexpress  $\alpha$ -synuclein in the rat nigrostriatal system. Rats were injected with rAAV2/8 expressing either human wild type (wt) or mutant  $\alpha$ -synuclein with S129 replaced by alanine (S129A) or aspartate (S129D). Contralateral substantia nigra injections containing empty vector served as control. Both wt and S129 mutants resulted in significant dopaminergic cell loss by 6 weeks, but only small decreases in nigrostriatal terminal density and tyrosine hydroxylase (TH) expression. There were no significant differences in dopaminergic cell loss, nigrostriatal terminal density, or TH expression between wt and S129 mutants. Furthermore, we did not observe any differences in  $\alpha$ -synuclein aggregate formation or distribution among wt and either S129 mutant. These findings contrast with those from previous studies, and suggest that both S129 phosphorylation mutants result in dopaminergic neurotoxicity similar to wt. Further study is needed to clarify the effects of these S129 mutants and  $\alpha$ -synuclein phosphorylation in mammalian systems.

# Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease

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## Running Title

$\alpha$ -Synuclein toxicity unaffected by S129 mutants

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**Abstract**

Lewy bodies are found in Parkinson disease and related disorders, and are extensively phosphorylated at Ser-129. Recent studies suggest that S129 phosphorylation may mediate  $\alpha$ -synuclein aggregation and neurotoxicity, but have had conflicting results. We used recombinant adeno-associated virus (rAAV) to overexpress  $\alpha$ -synuclein in the rat nigrostriatal system. Rats were injected with rAAV2/8 expressing either human wild type (wt) or mutant  $\alpha$ -synuclein with S129 replaced by alanine (S129A) or aspartate (S129D). Contralateral substantia nigra injections containing empty vector served as control. Both wt and S129 mutants resulted in significant dopaminergic cell loss by 6 weeks, but only small decreases in nigrostriatal terminal density and tyrosine hydroxylase (TH) expression. There were no significant differences in dopaminergic cell loss, nigrostriatal terminal density, or TH expression between wt and S129 mutants. Furthermore, we did not observe any differences in  $\alpha$ -synuclein aggregate formation or distribution among wt and either S129 mutant. These findings contrast with those from previous studies, and suggest that both S129 phosphorylation mutants result in dopaminergic neurotoxicity similar to wt. Further study is needed to clarify the effects of these S129 mutants and  $\alpha$ -synuclein phosphorylation in mammalian systems.

**Key Words**

Adeno-associated virus, dopamine, Lewy bodies, striatum, substantia nigra, tyrosine hydroxylase.

## Introduction

Parkinson disease (PD) is the most common neurodegenerative movement disorder and affects about 1% of the population over the age 65. The pathological hallmarks of PD are progressive loss of nigrostriatal dopamine neurons, swollen dystrophic neurites, and characteristic, filamentous intracellular inclusions, called Lewy bodies. These inclusions are not unique to PD, but are also found in related disorders, such as dementia with Lewy bodies and multiple system atrophy (1-3). A principle component of Lewy bodies is  $\alpha$ -synuclein; a soluble, 140-amino acid protein that is abundantly expressed in brain and enriched in synaptic terminals (4). Although its normal function remains unclear, several point mutations in the  $\alpha$ -synuclein gene (*PARK1/SNCA*) have been identified and linked to families with autosomal dominant parkinsonism (5-7). Gene duplication and triplication have also been linked to familial parkinsonism, and demonstrate a gene-dosage effect which correlates with earlier disease onset and symptom severity (8-10). The fact that Lewy bodies are also found in these familial forms of parkinsonism (11), further suggests a role for  $\alpha$ -synuclein in PD pathogenesis.

Abnormal folding, aggregation, and deposition of  $\alpha$ -synuclein may be key steps in the pathogenesis of sporadic and familial PD (12). *In vitro*  $\alpha$ -synuclein self-aggregates and fibrillizes, forming aggregates suggestive of those seen in Lewy bodies and Lewy neurites (13, 14). Identified missense mutations in  $\alpha$ -synuclein also result in accelerated fibril formation (15, 16) and oligomerization (17). Moreover, dose increases (duplication, triplication) of the  $\alpha$ -synuclein gene promote its aggregation and deposition in insoluble fractions, consistent with inclusion formation (18). Animal models of human  $\alpha$ -synuclein overexpression likewise demonstrate increased aggregates, neurotoxicity, and recapitulate features of PD (19-21).

Epigenetic factors, such as phosphorylation, also influence  $\alpha$ -synuclein fibrillization and aggregate formation. For example, the C-terminal portion of  $\alpha$ -synuclein contains several phosphorylation sites (22, 23) and some evidence suggests that Ser-129 phosphorylation accelerates  $\alpha$ -synuclein fibrillization and enhances neurotoxicity. S129 is extensively phosphorylated in brains from patients with both sporadic and familial PD (24, 25), as well as DLB (26). Although a small proportion (< 5%) of  $\alpha$ -synuclein is normally phosphorylated, nearly 90% of  $\alpha$ -synuclein in Lewy bodies is phosphorylated at S129. Furthermore, *in vitro* S129

phosphorylation enhances fibril formation (24). In transgenic flies expressing human  $\alpha$ -synuclein with mutation of S129 to either alanine (S129A) to prevent phosphorylation or aspartate (S129D) to mimic phosphorylation, blocking S129 phosphorylation suppresses dopamine neuronal loss, whereas S129D enhances  $\alpha$ -synuclein neurotoxicity (27). In addition, overexpression of the *Drosophila* G-protein-coupled receptor kinase 2 (GRK2), which selectively phosphorylates  $\alpha$ -synuclein at S129, results in enhanced neurotoxicity. Blockade of S129 phosphorylation (S129A) also increases inclusion formation and correlates with decreased  $\alpha$ -synuclein neurotoxicity, suggesting that aggregates may be neuroprotective and sequester potentially toxic  $\alpha$ -synuclein species. However, a recent study in rats found the opposite and demonstrated that the S129A mutant was more toxic than wild-type human  $\alpha$ -synuclein and associated with decreased intracellular aggregates, whereas the phosphomimic S129D appeared to be neuroprotective (28). Thus, whether or not phosphorylation at S129 plays a critical role in mediating  $\alpha$ -synuclein-induced dopaminergic neurotoxicity remains uncertain.

In this study we examine the effect of  $\alpha$ -synuclein S129 phosphorylation state on dopaminergic neurotoxicity in a rat model of PD. Previous studies have established that viral-vector based overexpression (using either recombinant adeno-associated virus, rAAV, or lentivirus) of human  $\alpha$ -synuclein in the rat nigrostriatal system results in progressive dopaminergic cell loss, synuclein-rich aggregates, and dystrophic neurites (29, 30). New rAAV serotypes are increasingly being used and show increased transduction efficiency and delivery of transgene in the nervous system over the commonly used rAAV2/2 (31-33). Here, we present new data using rAAV2/8 for delivery of  $\alpha$ -synuclein S129 phosphorylation mutants and show no significant differences in nigrostriatal dopaminergic toxicity or morphology of  $\alpha$ -synuclein aggregates among wt and S129A/D mutants.

## Materials and Methods

*Virus production.* Construction of rAAV vectors used to express human wild-type  $\alpha$ -synuclein is as previously described (34). Overlap extension PCR was used to generate  $\alpha$ -synuclein S129 mutants (S129A and S129D) using the mammalian pcDNA-Synuclein (human wt, genbank L08850) vector as template and previously designed primers (available by request). S129 mutant and wt  $\alpha$ -synuclein were then cloned into AAV-CBA-WPRE vector using the subclone pIRES2-

EGFP (Clontech, Mountain View, CA). We also constructed an empty vector, AAV-CBA-WPRE, with no transgene (kindly provided by Dr. Miguel Sena-Esteves, Massachusetts General Hospital, Boston, MA). Recombinant AAV2/8 virus was generated via tripartite transfection of the *cis*-transgene, packaging (*rep* and *cap*) genes, and helper plasmid into HEK 293A cells (Harvard Gene Therapy Initiative, Harvard Medical School). Viral particles were purified by iodixanol density gradient, isolated, and titered by dot blot hybridization. Final titers for virus were for  $\alpha$ -synuclein wt  $1.4 \times 10^{13}$  gc/mL, S129D  $1.4 \times 10^{13}$  gc/mL, S129A  $7.7 \times 10^{12}$  gc/mL, and empty vector  $1.16 \times 10^{14}$  gc/mL.

*Stereotaxic viral injections.* All animal protocols and procedures were approved by the MGH Subcommittee on Research Animal Care. The nigrostriatal system is essentially unilateral, thus each animal received bilateral stereotaxic injections of rAAV into the substantia nigra (SN). In most cases virus with empty vector was injected contralateral to virus encoding human wt or mutant S129A/D  $\alpha$ -synuclein. Sprague Dawley rats (300-350 g) were anesthetized, placed in a Kopf stereotax, and bilateral small skull holes were drilled to expose the dura over the injection sites. Coordinates for SN injections from bregma were AP -5.2, ML  $\pm 2.0$ , and DV -7.4 from the dural surface and targeted the central SN pars compacta. For each virus,  $1.4 \times 10^{10}$  gc in 2  $\mu$ L were injected at 0.2  $\mu$ L/min using a microinjection pump (Stoelting Co., Wood Dale, IL) with 10  $\mu$ L Hamilton syringe and 33-gauge needle. After injection the syringe remained in situ for 5 minutes before withdrawal.

*Tissue preparation and immunohistochemistry.* At 2 or 6 weeks post-injection, rats were deeply anesthetized and transcardially perfused with cold 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains from a subset of 6-week animals were removed without fixation, and the striatum and midbrain quickly dissected on ice, and snap-frozen in isopentane for use in dopamine content measurement and immunoblotting. Perfused brains were postfixed for 24 hours, then cryoprotected in 30% sucrose/PBS, and serially sectioned at 40  $\mu$ m on a sliding microtome into 12 wells (forebrain and midbrain separately blocked). Sections were collected and stored in cryoprotectant, 30% sucrose and 30% ethylene glycol in PBS, until processed and analyzed. Briefly, free-floating sections were rinsed with PBS, then treated 3-5 min. with 10% methanol and 3% H<sub>2</sub>O<sub>2</sub> to inhibit endogenous peroxidases,

permeablized with 0.3% Triton X-100 in PBS, and blocked in 5% normal goat serum. Coronal sections through the striatum and nigra were immunostained with primary antibodies to TH (1:10,000 dilution; Millipore, Billerica, MA) or  $\alpha$ -synuclein LB509 (1:1000 dilution; Zymed Laboratories, Inc., San Francisco, CA) overnight at 4 °C. After washing, immunostaining was visualized with either fluorescent secondary (1:200 dilution; Alexa Fluor 488, Molecular Probes, Eugene, OR; or Cy3, Jackson ImmunoResearch, West Grove, PA) or biotinylated secondary, followed by avidin-biotin (Vectastain Elite Kit), and 3,3'-diaminobenzidine reaction. Immunostained sections were washed, mounted on Superfrost slides, and coverslipped (GVA mount, Zymed Laboratories, Inc., San Francisco, CA; or Permount, Sigma Chemicals). In some cases, adjacent coronal sections were also Nissl stained with 0.05% cresyl violet per standard protocols.

*Microscopy and Stereology.* Immunostained sections were viewed using an Olympus BX51 microscope with brightfield and epifluorescence attachment. Photomicrographs were taken with an Olympus DP70 digital camera and adjusted only for suitable contrast and brightness. To maintain detail, wide-field images were photomontaged from high-power photos using Adobe Photoshop CS3. Double-label immunofluorescence images of cells were obtained using a Zeiss LSM510 confocal microscope system. Images were obtained with multi-tracking to minimize spectral overlap.

Nigrostriatal cell loss was assessed by counting TH-immunoreactive cells in the SN pars compacta, including the adjacent pars lateralis and caudal dense cell group ventral to the medial lemniscus and retrorubral area, using unbiased stereology according to the optical fractionator principle (35). At least 8 sections (each 240  $\mu$ m apart) through the SN for each case were analyzed and counted using the Olympus CAST Stereology System. Sampling intensity was sufficient for a coefficient of error for the counting protocol of less than 0.1.

*Striatal TH-terminal expression;* Semi-quantitative analysis of TH-expression in nigrostriatal terminals at 6 weeks post-injection virus was performed by measurement of optical density of immunofluorescence intensity scans using a Scan Array Express (Perkin Elmer, Boston, MA). All sections for analysis were handled, washed, and immunostained at the same time, using



common reagents to minimize interanimal variability. Striatal sections approximately 1 mm rostral to the decussation of the anterior commissure were immunostained with antibody to TH and fluorescent secondary-Cy3(Molecular Probes), and scanned using 543-laser excitation. Using ImageJ (NIH), mean gray value of fluorescence intensity within the dorsolateral striatum was obtained for each section and normalized to background, corpus callosum.

*Immunoblotting:* Striatal and midbrain tissue were separately suspended in 300  $\mu$ L lysis buffer (50 mM Tris-HCL, pH 7.4; 175 mM NaCl; 5M EDTA, pH 8.0; and protease inhibitor (Roche)) and homogenized on ice for 10-15 s. Each sample was centrifuged for 15 minutes at 4  $^{\circ}$ C, filtered, and then 1% Triton X-100 added to the lysate. After 1 hour incubation on ice, lysates were centrifuged for 60 min at 4  $^{\circ}$ C to collect the triton-X insoluble fraction. Triton-soluble lysate was separated and the insoluble pellet resuspended in 2% SDS-containing lysis buffer (Triton-insoluble fraction), then sonicated for 10 s. Protein concentration for each lysate was determined by BCA assay. Five micrograms of each sample was separated on a 10-20% Tris-glycine pre-cast gel (Invitrogen), transferred to PVDF, and immunoblotted for TH (mouse TH-2 antibody, Sigma) or  $\alpha$ -synuclein (mouse Syn1, BD Transduction Laboratories, or Syn (LB509), Zymed, antibody). All blots were immunostained for GAPDH (rabbit antibody, Abcam) as loading control. In some cases samples for each condition were pooled. Immunoblotted  $\alpha$ -synuclein, TH and GAPDH were detected with secondary antibody conjugated to HRP and reacted with ECL (GE Healthcare), per protocol. Films were scanned using a FluorChem system and analyzed with ImageJ software (NIH). TH and  $\alpha$ -synuclein content for each sample was normalized to GAPDH.

*Dopamine content:* Striatal tissue was thawed, homogenized, and mixed in lysis buffer with dihydrobenzylamine added as internal control. Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured by HPLC with electrochemical detection and normalized to protein content per sample (see 36).

*Statistics:* All data were expressed as group mean  $\pm$  SEM. Stereological estimates of nigral TH cell survival, mean striatal terminal density, and dopamine measurements were analyzed using

one-way ANOVA with Bonferonni's multiple comparison post-hoc (Prism GraphPad 4.03, San Diego, CA) unless otherwise stated.

## Results

We used the pseudotyped rAAV 2/8 for delivery of transgene, because recent rAAV serotype comparisons have shown increased transduction efficiency over that of rAAV2/2 (33, 36). Transgene expression for rAAV2/8 is also markedly enhanced over rAAV2/2 and rapidly reaches a steady-state by 2-4 weeks (37, 38). Rat substantia nigra (SN) were stereotactically injected with equal volume and genome copies ( $1.4 \times 10^{10}$  gc) of rAAV vector encoding either human wt or S129 phosphorylation mutant (S129A for substitution to alanine, or S129D for aspartate)  $\alpha$ -synuclein followed by an internal ribosomal entry site (IRES) and the reporter gene, EGFP (enhanced green-fluorescent protein) resulting in bicistronic expression. To control for possible viral toxicity and injection effects, the contralateral SN of several rats was injected with empty rAAV vector and used as an internal control. Based on preliminary experiments with rAAV2/8, its rapid transgene expression and spread, we selected 2 and 6 week time-points post-injection for analysis of wt and S129A/D  $\alpha$ -synuclein toxicity in the nigrostriatal system.

The pattern of transgene expression for all rAAV was similar. For  $\alpha$ -synuclein viruses, EGFP expression marked cells transduced with rAAV in the SN. All cells with EGFP co-expressed  $\alpha$ -synuclein as assessed by double immunofluorescence. Also, rAAV2/8 was neurotrophic; we did not observe transduction of glial elements. At 2 and 6 weeks post-injection, EGFP expression was widespread for  $\alpha$ -synuclein rAAV and included cells not only in the SN pars compacta (SNc), but also in the adjacent pars reticulata (SNr), ventral tegmental nucleus, and a portion of the ventral mesencephalic reticular formation (Figure 1). These findings are consistent with the reported enhanced transduction efficiency of rAAV2/8 compared to rAAV2/2 in the SN (33, 34, 37).

In addition to cellular expression of  $\alpha$ -synuclein within the SN, immunostaining for human  $\alpha$ -synuclein using the LB509 antibody (specific for amino acid residues 115-122) also demonstrated extensive transport of  $\alpha$ -synuclein to nigrostriatal terminals (Figure 2). Cases without extensive nigrostriatal labeling suggested failed or misplaced injections, and were

excluded from the analysis. Synuclein-positive terminals were widespread and found throughout the dorsal striatum (caudatoputamen), extending rostrally to the striatal pole and several millimeters caudal to the anterior commissure. These findings are consistent with the topographic distribution of nigrostriatal projections (39) and confirm transduction of a large proportion of SNc neurons for each case. Double labeling of SN with antibodies to  $\alpha$ -synuclein (or GFP) and tyrosine hydroxylase (TH) also demonstrated that rAAV injections transduced a majority of dopaminergic nigrostriatal neurons in the SNc and adjacent regions (data not shown).

*Viral expression.* To assess rAAV  $\alpha$ -synuclein expression levels for wt, S129A and S129D mutants, we performed Western blot analyses on striatal and midbrain extracts from 3 animals per group obtained at 6 weeks post-injection and compared these to extracts from the contralateral hemisphere injected with empty vector control. Blots from samples taken from wt, S129A, and S129D  $\alpha$ -synuclein cases probed with the human-specific LB509 synuclein antibody (Zymed/Invitrogen, Carlsbad, CA) demonstrated strong human  $\alpha$ -synuclein expression ipsilateral to the injection as expected. We also probed blots with the Syn-1 antibody (BD Transduction, San Jose, CA), which recognizes both endogenous rat and human  $\alpha$ -synuclein, to estimate the level of human  $\alpha$ -synuclein overexpression for each rAAV compared to endogenous levels (Figure 3). Comparison of the  $\alpha$ -synuclein injected side to the contralateral striatum or midbrain, empty rAAV vector control, revealed levels of  $\alpha$ -synuclein overexpression with a mean ratio (ipsilateral/contralateral side) of approximately twice that of endogenous rat  $\alpha$ -synuclein.

*Comparison of nigrostriatal toxicity.* Unbiased stereological estimates of TH-positive cells in the SN were performed at 2 and 6 weeks post-injection of rAAV expressing wt or mutant, S129A or S129D,  $\alpha$ -synuclein and compared to empty vector injections. In preliminary studies, at 2 weeks subtle TH cell loss was observed in both wt and mutant S129A and S129D groups ( $n = 2-3$  for each group). These findings were somewhat surprising given the early time point, but dopaminergic neurotoxicity for S129A has been reported as early as 4 weeks previously (28). Qualitative comparison TH-cell loss with cresyl violet stained sections confirmed proportional nigral cell loss and gliosis. Given the modest extent of cell loss at 2 weeks, we examined a larger cohort (6-10 per group) at 6 weeks to further compare toxicity of wt and S129 mutant  $\alpha$ -synucleins.

At 6 weeks post-injection, there was evident TH cell loss in the SNc for both wt and S129 mutant  $\alpha$ -synuclein groups (Figure 4). Cell loss, as opposed to TH-phenotype loss, was confirmed by analysis of Nissl stained sections and qualitative comparison with nigral TH cell loss. Stereological estimates of remaining TH cells in the SN demonstrated significant cell loss compared to empty vector control for wt ( $20.5 \pm 6.8\%$ ,  $p < 0.05$ ), S129A ( $29.9 \pm 2.4\%$ ,  $p < 0.001$ ), and S129D ( $27.0 \pm 6.7\%$ ,  $p < 0.01$ ) mutants ( $F[3,24] = 9.96$ ,  $p = 0.0002$ ) (Figure 5A). The mean TH-cell loss was 25.8% and ranged from 7-45% for all cases. Post-hoc analysis revealed no significant differences in TH cell counts between wt, S129A, or S129D. Qualitative comparison of nigral TH staining at 6 weeks also did not differ.

*Nigrostriatal TH and dopamine content.* Immunostaining of striatal sections for TH did not show nigrostriatal terminal loss at 2 weeks. By contrast, at 6 weeks TH-immunostaining revealed loss of terminal density in the dorsal striatum for both S129A and S129D cases compared to wt (Figure 2). Semi-quantitative analysis, using scan array measurement of immunofluorescence optical density, also revealed a small decrease in dorsal striatal TH+ terminal density for wt and mutant S129A and S129D cases relative to control, but these findings did not achieve statistical significance (Figure 5B). Relative side-to-side comparison in cases with internal empty vector control showed a similar trend of decreased dorsal striatal TH+ terminal density for S129A and S129D mutant cases, but also did not achieve significance.

To further investigate potential changes in striatal dopaminergic innervation, we also quantified striatal dopamine content at 6 weeks post-injection via HPLC fractionation and electrochemical detection. Dopamine was extracted from striatal tissue ipsilateral to the SN injection of either wt or mutant (S129A or S129D)  $\alpha$ -synuclein and compared to that extracted from the contralateral control striatum (SN injected with empty rAAV vector). In wt cases striatal dopamine content and levels of its metabolite, DOPAC, did not differ (Figure 5C). In contrast, relative dopamine content for S129A did significantly decrease, mean ratio (ipsilateral/contralateral striatum)  $0.66 \pm 0.04$  ( $p = 0.006$ , one sample t-test for hypothesized ratio of 1). Dopamine (DA) levels also decreased for S129D (mean ratio  $0.59 \pm 0.15$ ), but did not achieve statistical significance ( $p = 0.055$ ). Similarly, striatal DOPAC content for both S129A and S129D demonstrated a trend

toward decrease, but were not significant. These results are consistent with the loss of 25% of TH+ neurons in the SN.

*Synuclein toxicity, cellular aggregates, and terminal morphology.* Examination of sections stained for human  $\alpha$ -synuclein (LB509) at 2 and 6 weeks using brightfield and confocal microscopy revealed progressive changes in nigral cell morphology. Synuclein-positive cells in wt and mutant S129A and S129D cases were densely immunostained throughout the nucleus and cytoplasm (Figure 6). At 6 weeks a subset of cells contained synuclein-positive aggregates. Some larger cell aggregates co-stained for  $\alpha$ -synuclein and ubiquitin. There were no qualitative differences in aggregate size or distribution between wt and S129A or S129D cases. Transduced cells frequently had dystrophic process as previously described (29). Examination of nigrostriatal terminals immunostained for human  $\alpha$ -synuclein also showed dystrophic changes and neurites, but no qualitative differences were noted between wt, S129A or S129D.

## Discussion

We examined the role of  $\alpha$ -synuclein phosphorylation at Ser-129 by comparing the effects of overexpression of human wt and mutant  $\alpha$ -synuclein S129A (alanine blocks phosphorylation) and S129D (mimics constitutive phosphorylation) in the rat nigrostriatal system. A targeted viral vector-based approach with rAAV2/8 was used to overexpress  $\alpha$ -synuclein in the SNc. Neither Ser-129  $\alpha$ -synuclein mutant appeared to significantly alter dopaminergic nigrostriatal toxicity in the rat PD model. At 2 weeks, early neurodegenerative changes and TH cell loss were apparent, and by 6 weeks overexpression of wt and S129A and S129D mutants all resulted in significant (20-30%) nigral dopaminergic cell loss (confirmed on Nissl-stained sections) compared to empty vector control. However, there were no significant differences in dopaminergic nigrostriatal cell toxicity between wt, S129A, or S129D  $\alpha$ -synuclein at 6 weeks. Furthermore, the size and distribution of  $\alpha$ -synuclein aggregates did not differ among wt and either S129 mutant. Consistent with these findings, we did not detect major changes in striatal TH-terminal density or expression, although striatal dopamine content was decreased for S129A and S129D. The normal striatal dopamine levels for wt  $\alpha$ -synuclein are not surprising because over 6 weeks non-geriatric rodents show the ability to recover dopamine content after nigrostriatal lesion, such as that caused by MPTP (40). The lack of recovery of dopamine content for both S129 mutants thus

may suggest a decrease in functional neurochemical compensation or plasticity. Morphologically, though,  $\alpha$ -synuclein positive nigrostriatal terminals for S129 cases did not differ significantly from those of wt. However, S129 mutants may alter dopamine release and synaptic function as these are putatively regulated by  $\alpha$ -synuclein (41).

These findings contrast with those from previous studies in similar *Drosophila* and rat PD models that also compared wt  $\alpha$ -synuclein and S129A and S129D phosphorylation mutants (27, 28). In the fly, S129D was more toxic to dopamine cells and S129A was not only protective, but also associated with an increase in  $\alpha$ -synuclein aggregates. Recent data in rats found the opposite result and demonstrated increased toxicity for S129A and possible neuroprotective effects for S129D (28). Toxicity, though, was similarly associated with a decrease in large, insoluble  $\alpha$ -synuclein aggregates, whereas neuroprotection correlated with the presence of cytoplasmic inclusions. By contrast, we observed no differences in toxicity or aggregate formation for wt  $\alpha$ -synuclein or the phosphorylation mutants S129A and S129D in the rat.

Differences in study design may in part explain these differing results among studies. We used rAAV2/8 to deliver and overexpress  $\alpha$ -synuclein in the rat SN, whereas Gorbatyuk et al. (28) employed a different serotype, rAAV2/5. Both of these rAAV's show enhanced transduction efficiency (increase in cell number and volume transduced) over that of rAAV2/2 in the rodent nigrostriatal system (33, 38, 42). However, rAAV2/8 results in higher transgene expression than other rAAV serotypes, including 2/2 and 2/5 (37). One concern is that elevated levels of  $\alpha$ -synuclein expression from high-titer AAV2/8 injections may mask differences in toxicity between wt and the S129 mutants. We thus injected a moderate dose of each virus ( $1.4 \times 10^{10}$  gc), resulting in only 20-30% nigral TH cell loss at 6 weeks. Using a similar dose of AAV2/5, Gorbatyuk et al. reported 40-60% cell loss for both wt and S129A, albeit at 8 weeks post-injection. Moreover, Western blot estimates of human  $\alpha$ -synuclein expression compared to endogenous rat  $\alpha$ -synuclein were also lower (only 2-fold) than that previously reported. These findings suggest that the amount of virus injected and, indirectly, levels of  $\alpha$ -synuclein expression in our study did not “saturate” the system. The fact that we saw toxicity for S129D also argues that the viral dose was not too low, as previously it appeared less toxic than wt (28).

Another difference between studies is the length of viral incubation. The time-course for expression of the transgene is similar for both rAAV 2/5 and 2/8, although perhaps a bit more rapid for rAAV2/8 (38). Two and 6-week time points were thus chosen for analysis, and may have been too early to detect differences in toxicity among wt and S129 mutants. However, even at 4 weeks toxicity was noted for S129A by Gorbatyuk et al. (28) and was evident by 2 weeks in this study. Moreover, by 6 weeks wt and both S129 mutants showed significant dopaminergic cell toxicity.

Recent *in vitro* data on S129 phosphorylation and phosphomimics, S129D/E, may also help to explain differences in results from rat and fly PD models. S129 phosphorylation increases  $\alpha$ -synuclein conformational flexibility and appears to inhibit fibril formation. Although designed as phosphomimics, S129D/E do not exactly reproduce the effects of native phosphorylation (43). S129 phosphomimics only demonstrate local changes in conformational dynamics and *in vitro* form fibrils similar to wt, but not as rapidly as that of S129A. Consistent with these findings we found similar toxicity for wt and S129D  $\alpha$ -synuclein. Rapid fibrillization due to S129A presumably leads to increased  $\alpha$ -synuclein aggregate formation that is thought to sequester toxic species. This hypothesis is supported by reduced S129A toxicity and increased  $\alpha$ -synuclein inclusions seen in the fly (27). However, contradictory results for S129A are seen in the rat (28), possibly suggesting that while fibrils form, the S129A mutant in a mammalian system may also enhance formation of toxic  $\alpha$ -synuclein oligomers. Together, these findings emphasize the importance of phosphorylation at S129 as a regulator of  $\alpha$ -synuclein function, but do not fully clarify its effects on toxicity.

Increasing evidence suggests that large  $\alpha$ -synuclein aggregates may be neuroprotective and that S129 phosphorylation plays a role in aggregate formation (44). A high proportion of  $\alpha$ -synuclein in Lewy bodies is phosphorylated at S129 (24, 25). Furthermore, co-expression of human  $\alpha$ -synuclein and protein kinases, such as G-protein-receptor kinase 5 (GRK5)—one of many kinases that phosphorylate at S129 (27, 45)—is associated with  $\alpha$ -synuclein aggregate formation in cell culture (46). However, recent data indicate that S129 phosphorylation can also inhibit fibril formation and  $\alpha$ -synuclein oligomerization, but with longer incubation times, fibrillary aggregates such as those found in Lewy bodies eventually form (43). Lingering questions remain



though, including what influences  $\alpha$ -synuclein phosphorylation, when it occurs (before, during or after fibrillization and aggregate formation), and how it may mediate neurotoxicity.

Several kinases phosphorylate  $\alpha$ -synuclein at S129, including casein kinases 1 and 2, and GRK2 and 5 (22, 45, 47), but specific phosphatases have yet to be identified. Regulation of  $\alpha$ -synuclein phosphorylation state *in vivo* thus remains unclear, in particular due to the multiple cellular compartments in which  $\alpha$ -synuclein is found. Phosphorylation of  $\alpha$ -synuclein by GRKs is enhanced by phospholipids (45). Liposomal binding of  $\alpha$ -synuclein and GRK appears to increase its kinase activity (48).  $\alpha$ -Synuclein phosphorylation by casein kinase 2 is similarly enhanced by liposomes. There is also evidence for  $\text{Ca}^{2+}$ /calmodulin interaction with GPK5 and activation of  $\alpha$ -synuclein phosphorylation (49). Further study remains, though, to identify other specific kinases and protein and membrane interactions involved in  $\alpha$ -synuclein phosphorylation.

The function of  $\alpha$ -synuclein remains largely unknown and further complicates our understanding of its role in PD pathophysiology. Putative functions include regulation of synaptic dopamine transport (41), vesicle formation and trafficking (51), mitochondrial function (41, 50), and nuclear transcription (51). How  $\alpha$ -synuclein phosphorylation affects these functions is unclear, but disruption in any one of these could lead to dysfunction and potential neurotoxicity. Findings presented here, though, suggest that phosphorylation state at S129 and its effects *in vivo* are more complex than previously thought, and alone, may not play as significant a role in mediating dopaminergic toxicity. A better understanding of  $\alpha$ -synuclein phosphorylation and aggregate formation is clearly needed and will help target novel therapies.



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## Figure Legends

**Figure 1.** Fluorescence immunostaining of SN injected with rAAV2/8 expressing wt  $\alpha$ -synuclein and (ires-)EGFP. Tyrosine hydroxylase (TH) staining shows the distribution of dopaminergic SN neurons at representative rostral (**A-C**) and caudal (**D-F**) levels. GFP+ transduced cells are found widely throughout the SN and almost exclusively co-localize with TH+ neurons. Bar = 250  $\mu$ m.

**Figure 2.** Striatal photos show the distribution of nigrostriatal terminals immunostained for TH and human  $\alpha$ -synuclein (LB509) at 6 weeks. Columns 1 and 2 compare striatal TH terminal labeling after empty vector control (**A, D, G**) and  $\alpha$ -synuclein (**B, E, H**) injection in the same animal. There is evident striatal TH terminal loss in the dorsal striatum ipsilateral to  $\alpha$ -synuclein injection that appears more prominent for both S129 mutants than for wt. However, the distribution of  $\alpha$ -synuclein-positive (LB509 antibody) striatal terminals (**C, F, I**) was similar among wt and mutant cases. Insets reveal high-power detail of  $\alpha$ -synuclein+ nigrostriatal terminals. Bars indicate respective magnification ( $\mu$ m).

**Figure 3.** Measurement of  $\alpha$ -synuclein and TH expression in midbrain tissue 6 weeks post viral injection. Western blots show samples from midbrain ipsilateral to  $\alpha$ -synuclein (wt, S129A, and S129D) injection and are compared to protein extracts from the contralateral midbrain injected with empty vector (–) control in the same animal. Five micrograms of protein from the left and right midbrain samples were Western blotted with primary antibody to  $\alpha$ -synuclein and TH, followed by secondary HRP-conjugated antibody and ECL detection. Bands were quantified and normalized to GAPDH loading control. The ratio of  $\alpha$ -synuclein and TH for the  $\alpha$ -synuclein (ipsilateral) versus empty vector (contralateral) side was calculated for each animal. Blots show  $\alpha$ -synuclein overexpression for most animals, but variable expression was evident as shown for wt. Decrease in TH expression is likewise shown for wt and S129 mutants.

**Figure 4.** Photomicrographs of representative SN sections from cases at 6 weeks demonstrate neurodegenerative changes and TH cell loss. **A-D** case 98, wt  $\alpha$ -synuclein. **E-H** case 99, S129A. **I-L** case 100, S129D. **A, E, I** show TH immunostaining in the contralateral SN after empty

vector control injection. **B, F, J** illustrate TH cell loss in the SN for wt and S129 mutant cases. **C, G, K** show the distribution of human  $\alpha$ -synuclein (LB509) expression (gray-black immunostaining) within the SN. **D, H, L** are adjacent sections stained with cresyl violet which demonstrate SN cell loss and gliosis at rAAV injection sites. Bar = 250  $\mu$ m.

**Figure 5.** Graphs of nigral TH cell loss, striatal TH terminal density, and dopamine content. **A**, Stereological estimates of TH cell loss in the SN at 6 weeks are expressed relative to empty vector control. Expression of both wt and S129 mutant  $\alpha$ -synuclein resulted in significant TH cell loss (mean 25.8%) compared to control (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ;  $F[3,24] = 9.96$ ,  $p = 0.0002$ ). However, TH cells did not differ among wt and S129A and S129D groups. **B**, Optical density scan array analysis of striatal TH terminal density showed a 16-25% decrease for wt and S129 mutants, but no significant difference among groups ( $F[3,27] = 2.514$ ,  $p = 0.080$ ). **C**, Relative striatal dopamine (compared to contralateral striatum injected with empty vector control), but not DOPAC content was reduced for S129 mutants only.

**Figure 6.** High-power photomicrographs of human  $\alpha$ -synuclein (LB509) immunostaining of nigral neurons in wt (**A-C**), S129A (**D-F**), and S129D (**G, H**) cases. Arrows mark large intracytoplasmic  $\alpha$ -synuclein+ aggregates. Most aggregates were either perinuclear or in the periphery (**A** and **G**). **B** shows typical nuclear ghosting, consistent with neurodegenerative change. Dystrophic neurites in **C** have characteristic  $\alpha$ -synuclein+ inclusions (arrowheads). Bar = 5  $\mu$ m.



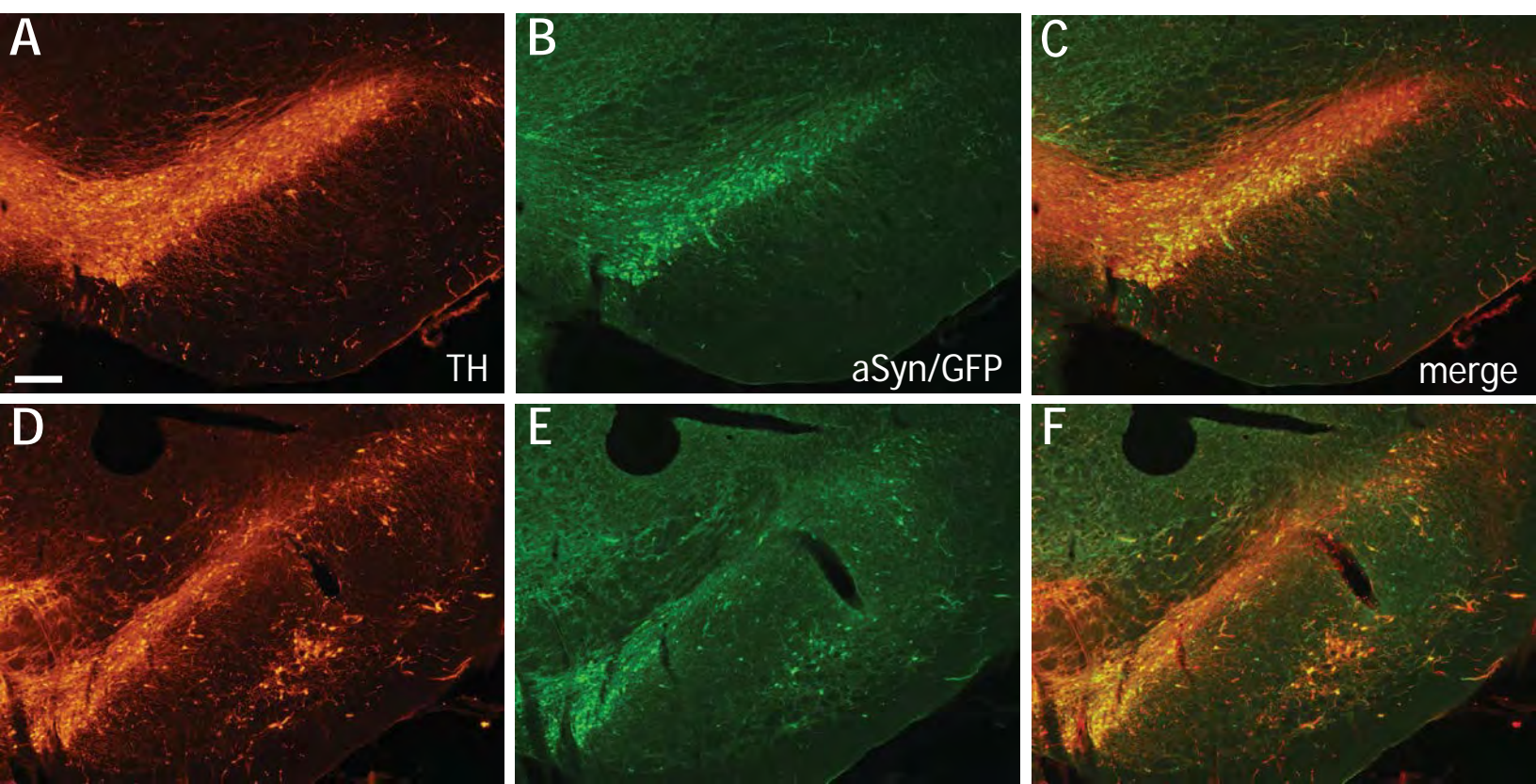


Figure 2

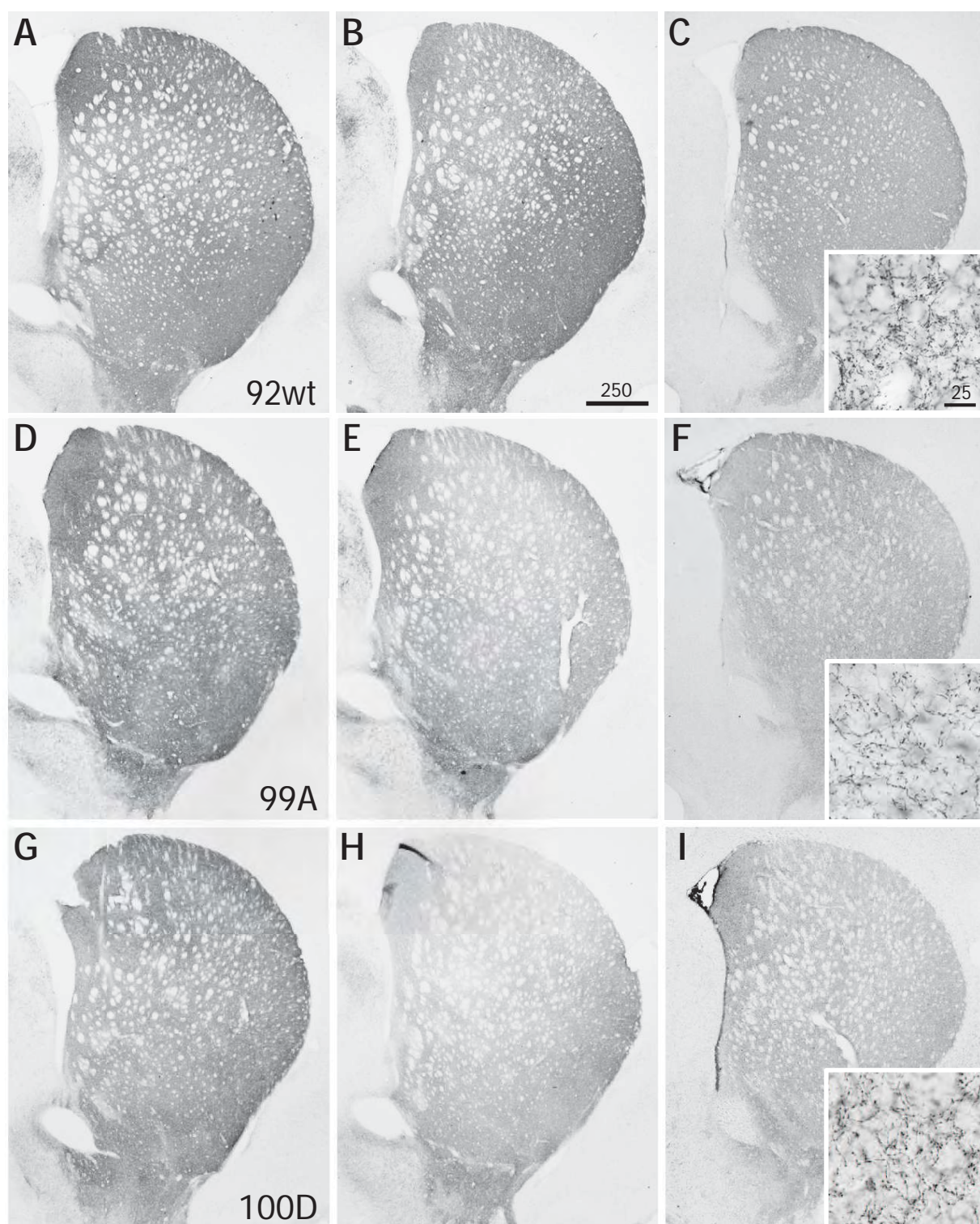




Figure 3

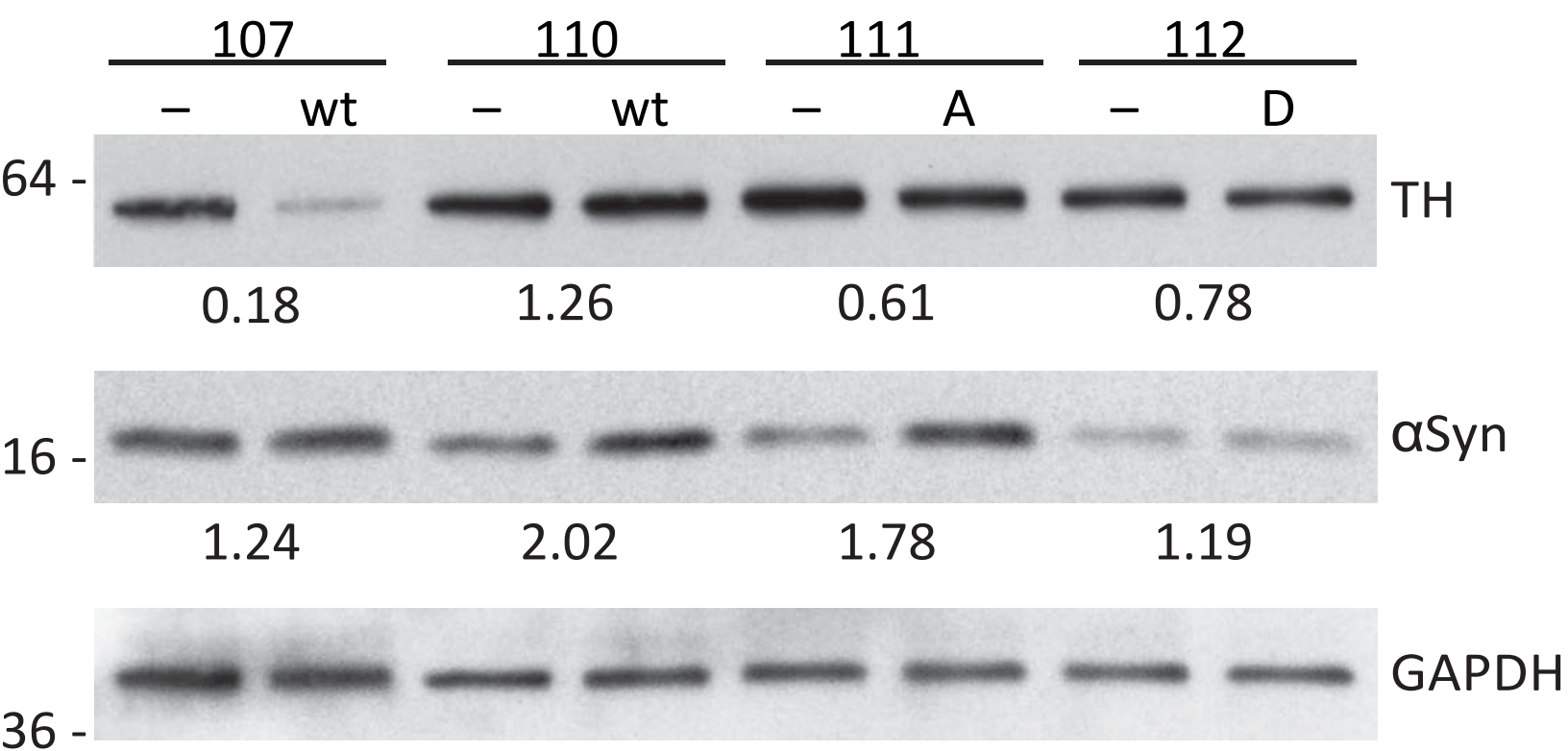
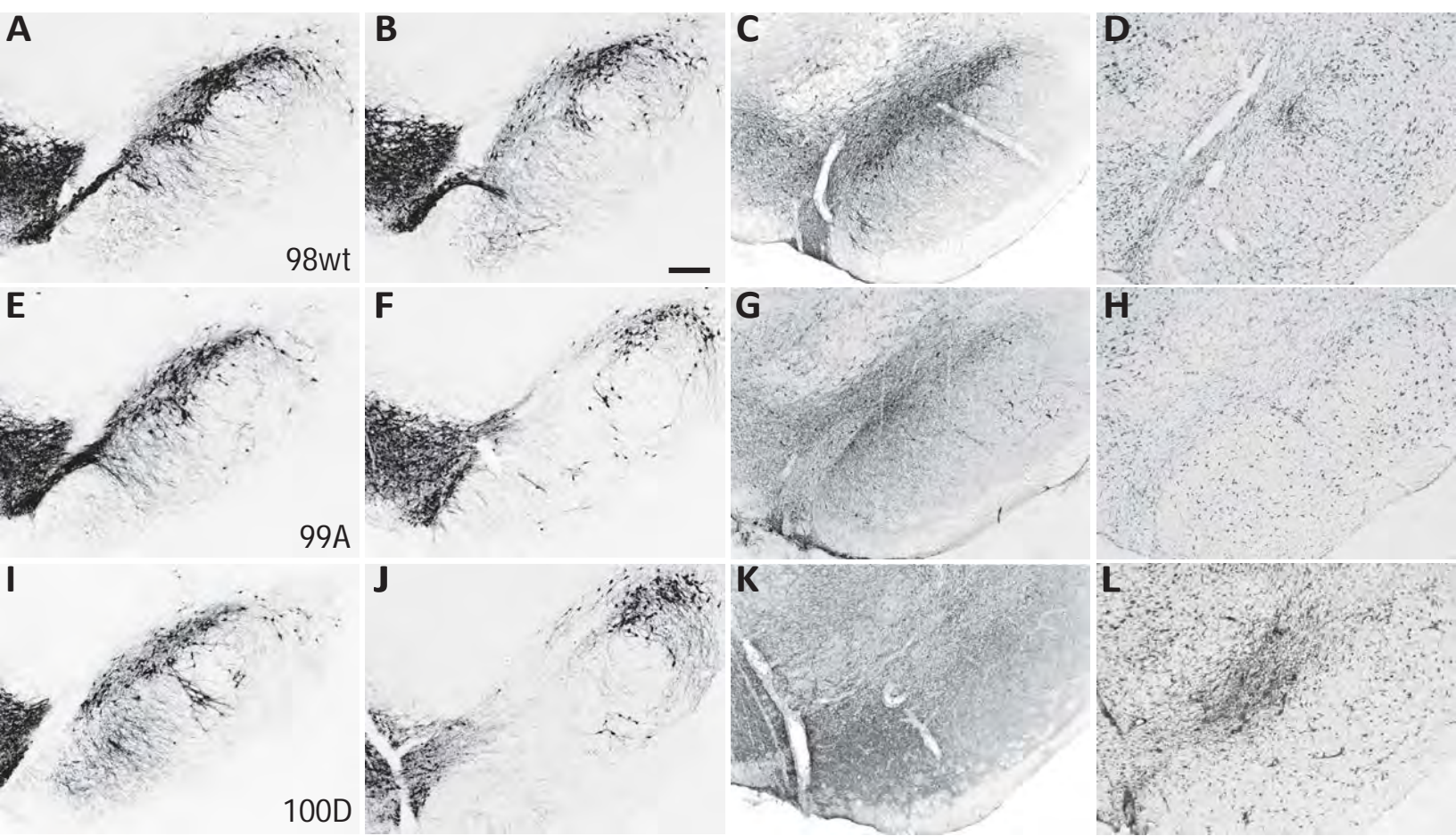


Figure 4



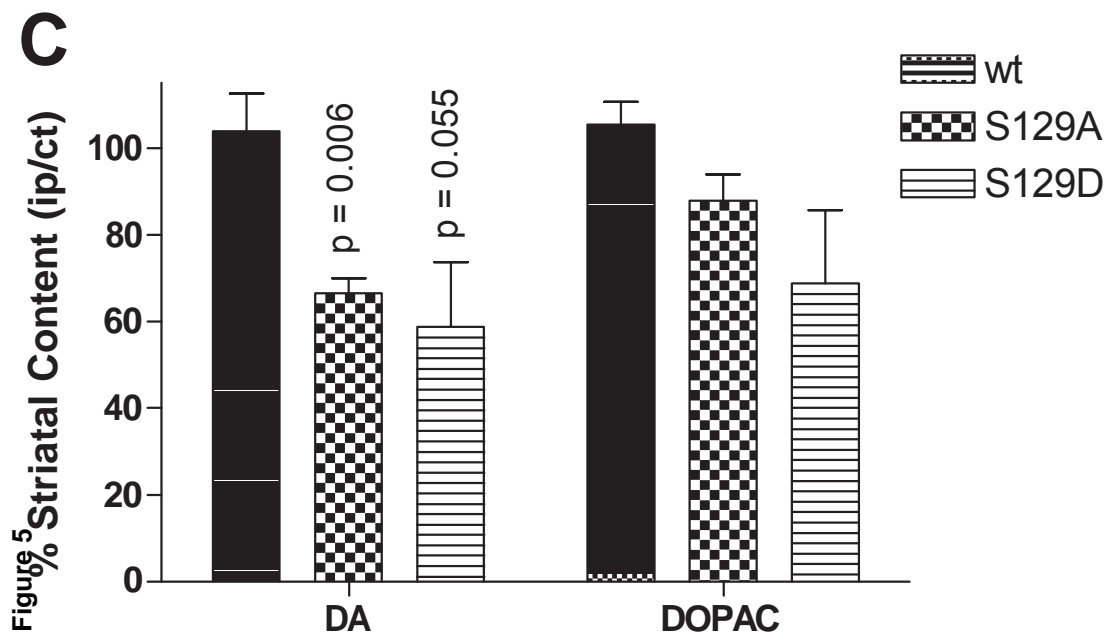
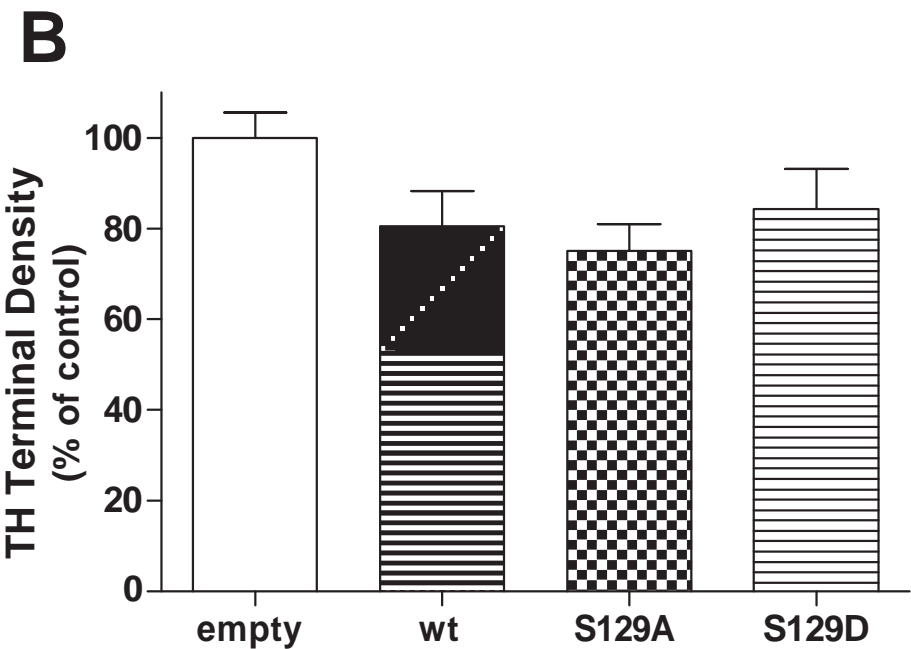
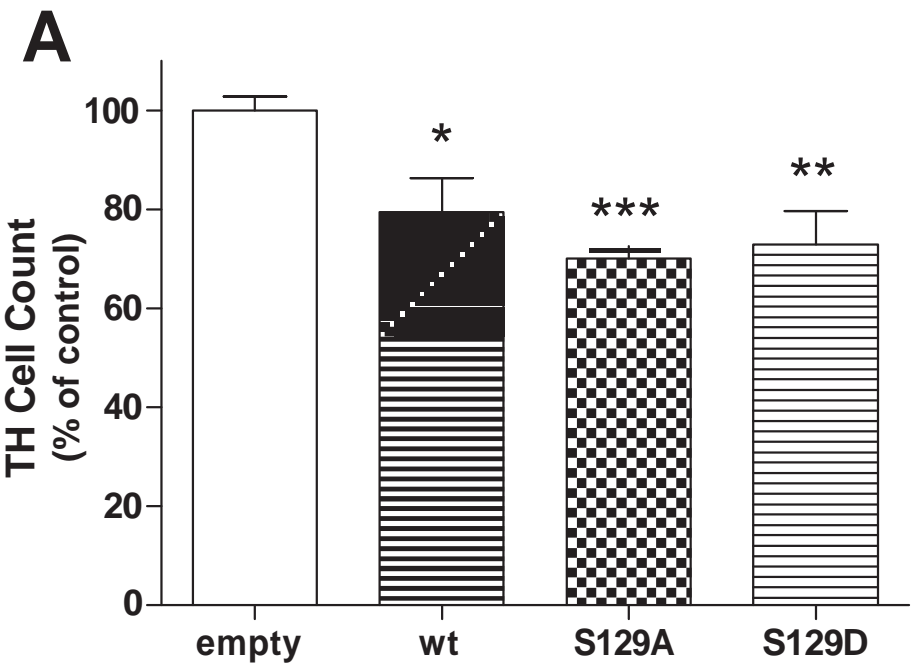
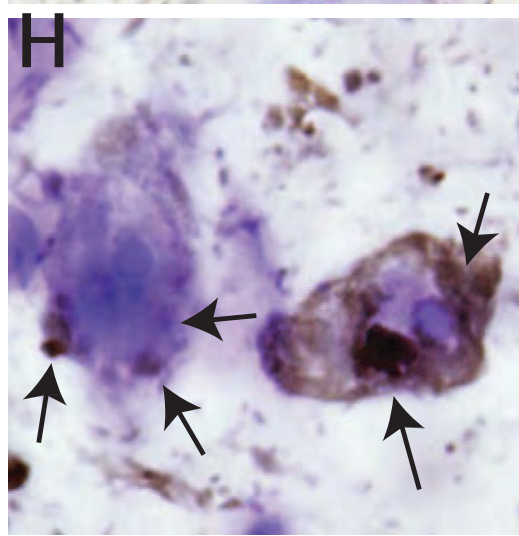
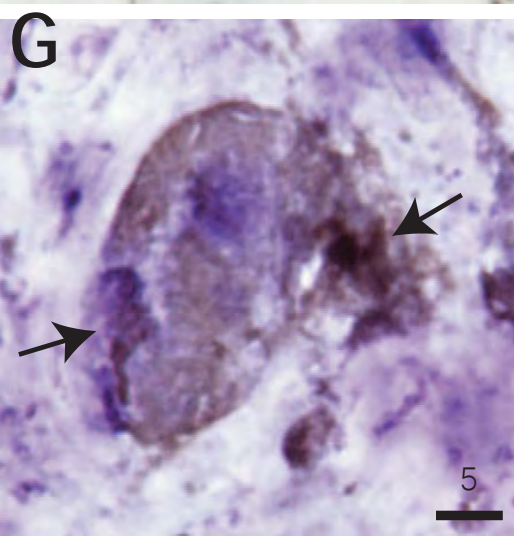
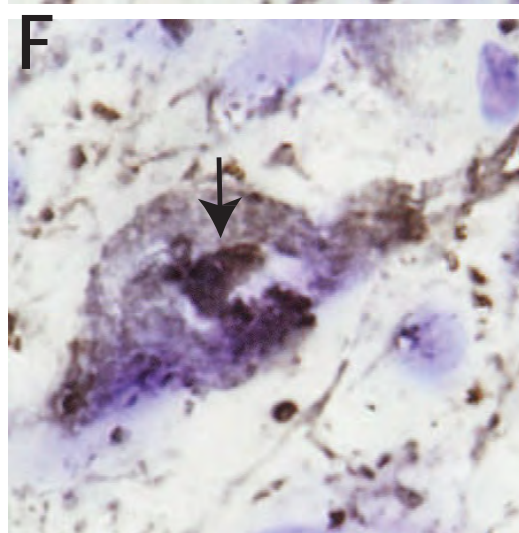
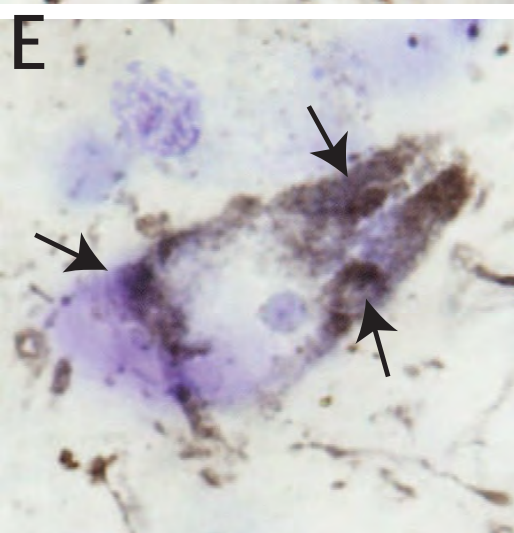
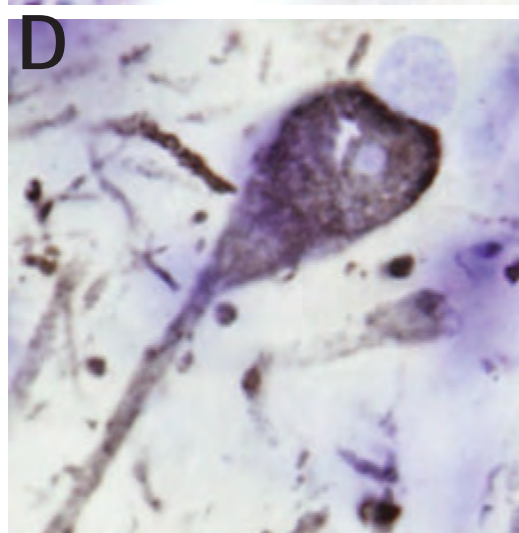
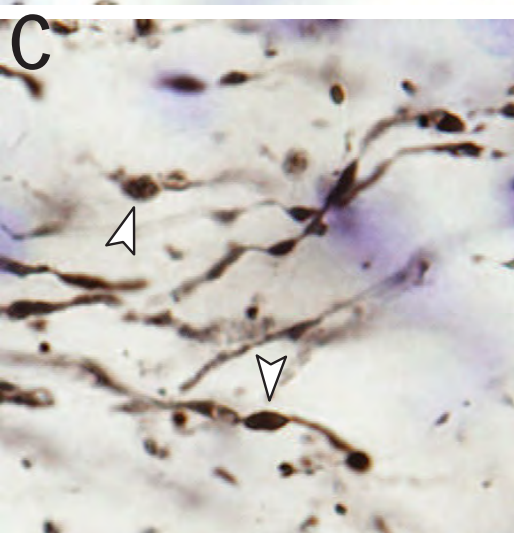
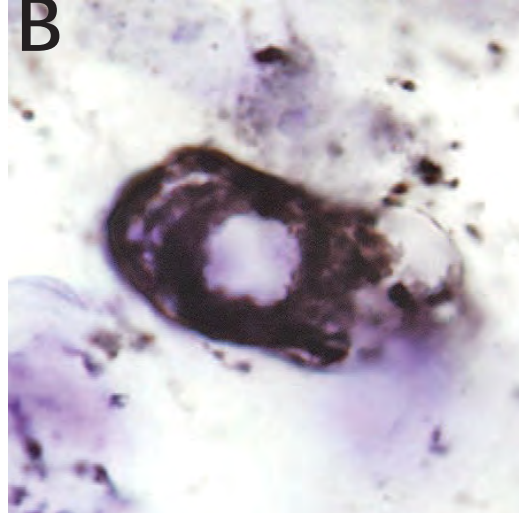
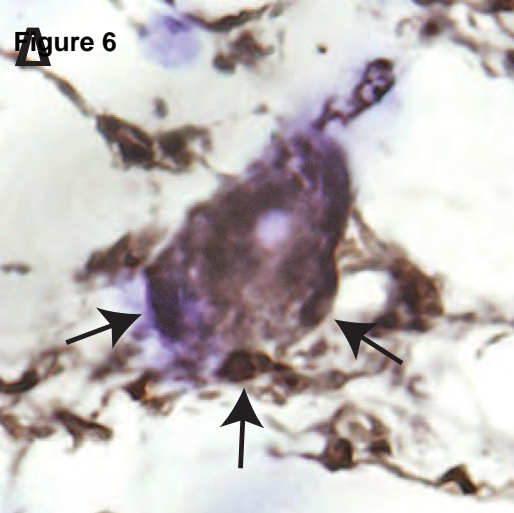
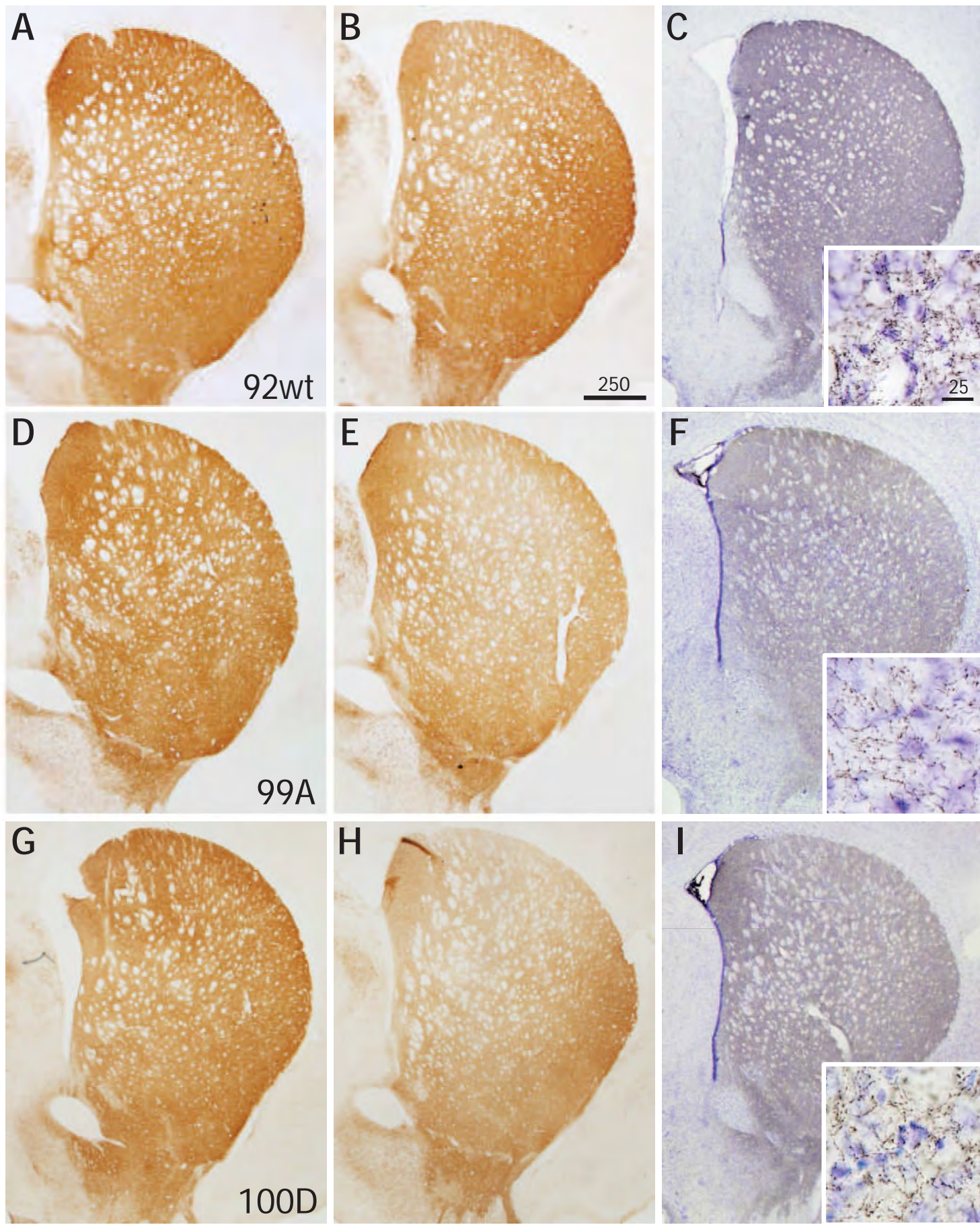




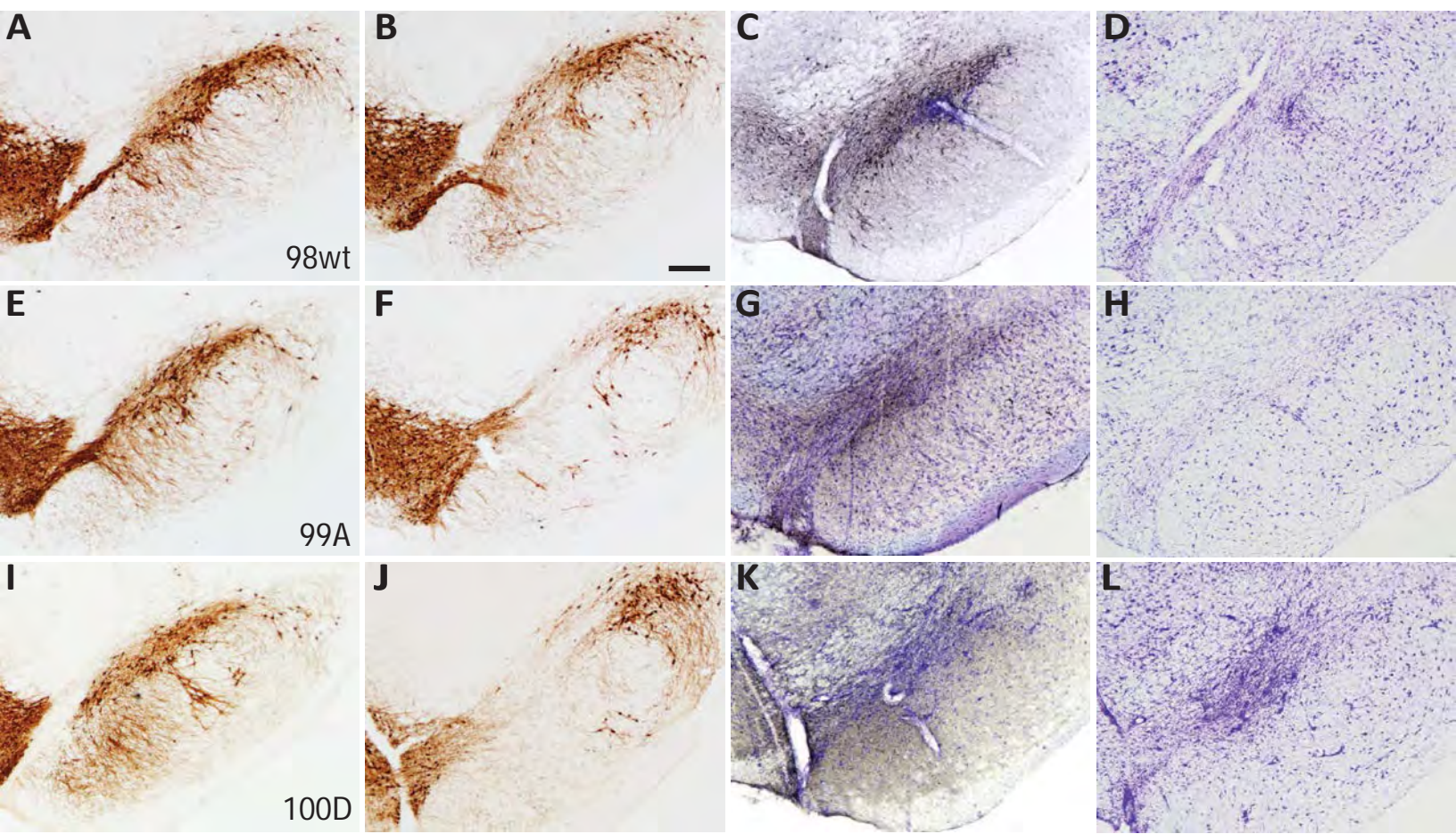
Figure 6











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## Presentation Abstract

Program#/Poster#: 142.5/F14

Title: Adenosine  $A_{2A}$  receptor gene deletion confers protection of nigral neurons in an  $\alpha$ -synuclein model of Parkinson's disease

Location: South Hall A

Presentation Time: Sunday, Oct 18, 2009, 8:00 AM - 9:00 AM

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Abstract: Parkinson's disease (PD), a progressive neurodegenerative disorder, results from the interaction of genetic and environmental factors in association with aging. From a genetic standpoint human  $\alpha$ -synuclein (*h- $\alpha$ SYN*) has been implicated in the PD phenotype. Mutations in the (*h- $\alpha$ SYN*) gene (e.g., A53T and A30P) may cause autosomal dominant familial PD. Transgenic mice expressing a *h- $\alpha$ SYN* gene with these two mutations (*hm<sup>2</sup>- $\alpha$ SYN* mice) develop age-related reductions in dopaminergic nigral neurons, striatal dopamine (DA) and locomotor activity. Pharmacological blockade of the adenosine  $A_{2A}$  receptor using caffeine (non-specific  $A_{2A}$  antagonist) and specific antagonists, as well as genetic elimination of the  $A_{2A}$  receptor are all neuroprotective in mouse models of PD. The present study sought to determine whether deletion of the  $A_{2A}$  receptor ( $A_{2A}^{-/-}$  mice) would afford neuroprotection in *hm<sup>2</sup>- $\alpha$ SYN* mice. Heterozygous  $A_{2A}^{+/-}$  mice were mated with  $A_{2A}^{+/-}$  mice that were also transgenic for

the wild-type (*hw- $\alpha$ SYN*) or doubly mutated (*hm<sup>2</sup>- $\alpha$ SYN*) form of the human synuclein gene under control of a 9-kb rat tyrosine hydroxylase (TH) promoter - all in the C57B/6J background. Offspring were sacrificed at 20-24 months of age. Striatal DA and metabolites were measured by HPLC-ECD. Stereological counts were performed in the substantia nigra pars compacta. Alternate brain sections collected were also stained using double immunohistochemistry for both TH-positive neurons and synuclein to assess the levels of *h- $\alpha$ SYN* in nigral dopaminergic neurons from brain sections of *A<sub>2A</sub>*<sup>[+/+]</sup> and *A<sub>2A</sub>*<sup>[-/-]</sup> *hm<sup>2</sup>- $\alpha$ SYN* male mice. In the presence of a functional *A<sub>2A</sub>* receptor, stereological counts were significantly lower (by approx 43%) in *hm<sup>2</sup>- $\alpha$ SYN* mice compared to either *hw- $\alpha$ SYN* or non-transgenic (NT) mice. By contrast, in *A<sub>2A</sub>*<sup>[-/-]</sup> mice the *hm<sup>2</sup>- $\alpha$ SYN* transgene did not reduce neuronal cell counts compared to either control. Striatal DA data showed a profile similar to that observed with the stereological counts. No differences were observed in the expression levels of *hm<sup>2</sup>- $\alpha$ SYN* immunoreactivity between the two *A<sub>2A</sub>* genotypes, indicating that a reduction in *hm<sup>2</sup>- $\alpha$ SYN* expression does not account for the reduced toxicity observed in the *A<sub>2A</sub>*<sup>[-/-]</sup> *hm<sup>2</sup>- $\alpha$ SYN* mice. Thus the *A<sub>2A</sub>* receptor appears to be required for neurotoxicity in this  $\alpha$ -synuclein mouse model of PD, supporting the neuroprotective potential of caffeine and more specific *A<sub>2A</sub>* antagonists in the chronic dopaminergic neuron degeneration that characterizes PD.

Disclosures: **A. Kachroo**, None; **E.K. Richfield**, None; **M.A. Schwarzschild**, None.

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**Deletion of adenosine A<sub>1</sub> or A<sub>2A</sub> receptors or treatment with caffeine  
reduces levodopa-induced dyskinesia in a model of Parkinson's disease**

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Running title: Adenosine receptors in L-DOPA-induced dyskinesia

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**Abstract**

Adenosine A<sub>2A</sub> receptor antagonism provides a promising approach to developing nondopaminergic therapy for Parkinson's disease (PD). Initial clinical trials of A<sub>2A</sub> antagonists have targeted PD patients with L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) in an effort to improve parkinsonian symptoms. The role of adenosine in the development of LID is little known, especially regarding its actions via A<sub>1</sub> receptors. We examined the effects of genetic deletion and pharmacological blockade of A<sub>1</sub> and/or A<sub>2A</sub> receptors on the development of LID, on the induction of molecular markers of LID including striatal preproenkephalin (PPE), and on the integrity of dopaminergic nigrostriatal neurons in hemiparkinsonian mice. Following a unilateral 6-hydroxydopamine (6-OHDA) lesion A<sub>1</sub>, A<sub>2A</sub> and double A<sub>1</sub>-A<sub>2A</sub> knockout (KO) and wild-type (WT) littermate mice, or caffeine- (and saline-) pretreated mice, were treated daily for 18-21 days with a low dose of L-DOPA. Total abnormal involuntary movement (AIM, a measure of LID) was significantly attenuated in all KOs and in caffeine-pretreated mice ( $p < 0.05$ ). An elevation of PPE mRNA ipsilateral to the lesion in WT mice was reduced in all KO mice. In addition, neuronal integrity assessed by striatal dopamine content was similar in all KO and caffeine-pretreated mice following 6-OHDA lesioning. Our findings raise the possibility that A<sub>1</sub> as well as A<sub>2A</sub> or combined antagonists may also confer a disease-modifying benefit of reduced *risk* of disabling LID. Furthermore, the report may strengthen the rationale for conducting trials of adenosine antagonists as initial rather than late adjunctive therapy for PD.

## Introduction

Blockade of adenosine A<sub>2A</sub> receptors is being attempted as a non-dopaminergic alternative treatment of Parkinson's disease (PD). In particular, the usefulness of A<sub>2A</sub> receptor antagonism to treat PD complicated by L-DOPA-induced dyskinesia (LID) has been studied in both animal models and clinical trials (Chen, 2003; Morelli et al., 2007). The rationale behind the use of A<sub>2A</sub> antagonists for symptomatic benefit with reduced risk of adverse effects in PD and LID depends inter alia on the discrete CNS distribution and colocalization of the A<sub>2A</sub> receptor with D<sub>2</sub> receptors in the 'indirect' pathway of the basal ganglia motor circuitry (Ferre et al., 1997; Kase, 2001; Fredholm et al., 2003). Experimentally it has been shown that elimination or blockade of A<sub>2A</sub> receptors expressed by forebrain neurons attenuate LID and related behaviors in hemiparkinsonian rodents or parkinsonian non-human primates (Bibbiani et al., 2003; Xiao et al., 2006).

However, adenosine also activates the adenosine A<sub>1</sub> receptor, which – in contrast to discretely expressed A<sub>2A</sub> receptor – is widely distributed throughout the CNS including the hippocampus and cortex as well as on the striatal neurons of the 'direct' pathway of the basal ganglia (Fastbom et al., 1987; Johansson et al., 1997; Tohyama, 1998). It has been proposed that blocking A<sub>1</sub> receptors on striatonigral neurons of the direct pathway may facilitate motor activity by disinhibiting the motor stimulant actions of colocalized dopamine D<sub>1</sub> receptors on these neurons, whereas blocking A<sub>2A</sub> receptors on striatopallidal neurons of the indirect pathway may produce a parallel behavioral activation by inhibiting the motor suppressant actions of colocalized D<sub>2</sub> receptors on these neurons (Ferre et al., 1997). However, A<sub>1</sub> receptors are as abundant in



striatopallidal and striatonigral neurons (Ferre et al., 1996) and a selective action is not easily conceived.

Thus adenosine may modulate LID through opposing or cooperative actions on two of its receptors in the CNS. To explore the roles of these receptors alone and in combination in a mouse model of LID in PD, we characterized single A<sub>1</sub> and A<sub>2A</sub> KO, as well as double A<sub>1</sub>-A<sub>2A</sub> receptor KO phenotypes in hemiparkinsonian mice that are all littermates of double heterozygote crosses in a congenic C57Bl/6 background. In addition to this genetic approach to addressing adenosine receptor involvement in LID, we investigated the effect of pharmacological blockade of adenosine receptors using the widely consumed non-specific adenosine antagonist caffeine. The investigation of caffeine was also prompted by preliminary clinical data that raised the possibility of a link between higher levels of caffeine consumption among PD patients and a reduced risk of subsequent dyskinesia development (Schwarzschild et al., 2003). We chose both a moderate dose of caffeine, which elicits hyperlocomotion (15 mg/kg), and a low dose of caffeine (3 mg/kg), which is capable of modifying neuroplasticity (as in that of conditioning preference) with little accompanying motor stimulant effect (Fredholm et al., 1999).



## Materials and methods

### Generation of adenosine receptor KO mice

Wild-type (WT) control ( $A_1^{+/+}, A_{2A}^{+/+}$ ),  $A_1$  KO ( $A_1^{-/-}, A_{2A}^{+/+}$ ),  $A_{2A}$  KO ( $A_1^{+/+}, A_{2A}^{-/-}$ ) and  $A_1$ - $A_{2A}$  double KO ( $A_1^{-/-}, A_{2A}^{-/-}$ ) mice were generated by double heterozygous mating ( $A_1^{+/-}, A_{2A}^{+/-}$  x  $A_1^{+/-}, A_{2A}^{+/-}$ ) and genotyped by PCR analysis of tail DNA as described previously (Bastia et al., 2005; Kachroo et al., 2005; Xiao et al., 2006). The double heterozygotes were the offspring of crosses between homozygous  $A_1$  KO and homozygous  $A_{2A}$  KO mice from lines that we rendered congenic for the C57Bl/6 strain background (Banbury Conference, 1997).

### 6-OHDA lesion of striatum

All mice were maintained in home cages with a 12 h light/dark cycle. All experiments were performed in accordance with the Massachusetts General Hospital and National Institutes of Health guidelines on the ethical use of animals. The dopaminergic nigrostriatal pathway on the left side of each mouse was lesioned by a stereotactic intrastriatal infusion of 10  $\mu$ g 6-OHDA (hydrobromide) as described previously (Fredduzzi et al., 2002; Xiao et al., 2006). The mice were fed with both veterinary (Nutri-Cal) and human infant (Enfamil) nutritional supplements immediately after surgery for several days, followed by soft food (Nutra-Gel Diet) to help ensure survival. Twelve days following surgery each mouse underwent a cylinder test (Cenci and Lundblad, 2007), in which asymmetry of forepaw placement on the inner wall of a plexiglass cylinder was used as an indirect assessment of lesion extent. Mice with <85% of dopamine loss (~15% of the lesioned mice) in the lesioned striatum (compared to the contralateral, non-lesioned

striatum) were excluded from all data analysis (except for that of dopamine content). The mice included in the final analyses of the KO experiment comprised 29 male and 9 female mice (3-14 months old with average age 7.5 months) and were balanced for age, weight and gender across genotypes (n=8-12 in each genotype group).

### **Pharmacological treatment and behavioral testing**

Two weeks after 6-OHDA lesioning, mice were treated daily with L-DOPA (freshly prepared, 2 mg/kg ip) for 18-21 days. In the adenosine receptor KO experiment, benserazide (2 mg/kg ip in saline) was administered 20 min prior to each dose of L-DOPA. In the adenosine antagonist experiment, 20 mice were divided into three groups, saline (n=7), caffeine (3 mg/kg ip, n=6) and caffeine (15 mg/kg, ip, n=7) groups (balanced for performance on the cylinder test and home cage residence), in which each daily L-DOPA (mixed with benserazide 2 mg/kg in saline) was preceded by saline or caffeine by 10 min. Behaviors (AIMs and rotations) were recorded every 2-3 days as described previously (Xiao et al., 2006).

### **Molecular and neurochemical assessments**

Three days after the last L-DOPA injection for both KO and caffeine experiments, whole brains were dissected out. The rostral half of the striata was used for mRNA expression quantification of PPE and prodynorphin (PPD) by *in situ* hybridization, as described previously (Benn et al., 2004; Xiao et al., 2006). The probe sequence (5' to 3') for PPE mRNA was as follows: ATC TGC ATC CTT CTT CAT GAA ACC GCC ATA CCT CTT GGC AAT GAT CTC. The probe sequence (5' to 3') for PPD mRNA was as follows: ATG GGG GCT TCC TGC GGC GCA TTC GCC CCA AGC TGA AGT GGG

ACA. Quantitative densitometric analysis of the mRNA signals was performed using Image J 1.40g software (National Institutes of Health, USA). Optical density (OD) of the transcripts was quantified for the striatum and expressed as the ratio on the 6-OHDA-lesioned side to that on intact (contralateral) side.

The remaining frozen brains containing the caudal half of the striata were sectioned to a thickness of 400  $\mu\text{m}$  at  $-17^{\circ}\text{C}$ , and the striata were micropunched (Stoelting, 1.25 mm diam). The cores from the left and right striata were analyzed by HPLC with electrochemical detection for dopamine (DA) and DOPAC content (Chen et al., 2001; Xu et al., 2006).

### **Statistical analysis**

All data are expressed as group average  $\pm$  SEM. The difference between treatments (WT versus KO; caffeine versus saline) during the plateau phase of LID (from day 11 on for each analysis) was evaluated using a repeated measures model with a compound symmetry covariance structure. No significant time by treatment interaction was observed in any model, so only the main effect of group is reported. If a global difference between groups was observed, post-tests were performed and Holm's correction was used to account for multiple comparisons (Holm, 1979). A Wilcoxon signed rank test was used to compare each the plateau measurements to the day 1 measurements for each genotype. The analysis was generated using SAS software, Version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA.) and GraphPad. Student's *t*-test was used for the remaining statistical analyses of molecular

correlates and dopamine loss (Tables). A value of  $p < 0.05$  was considered to be significant.

## Results

### Attenuation of behavioral sensitization in adenosine receptor knockout mice

Following daily L-DOPA treatment, the hemiparkinsonian mice developed behavioral sensitization, as recorded by contralateral rotations, and dyskinesia, quantified by abnormal involuntary movement (AIMs) scale (Figure 1). Acutely (on day 1), responses to L-DOPA were indistinguishable between adenosine receptor genotypes. Chronically, rotational sensitization on the plateau phase (day 11-21) in A<sub>1</sub> KO, A<sub>2A</sub> KO or A<sub>1</sub>-A<sub>2A</sub> double KO mice was attenuated over the 21 days ( $p=0.12$ , for each comparison, Holm's corrected  $p$ -values) in comparison to WT (Figure 1A). Total AIMs in A<sub>1</sub> KO, A<sub>2A</sub> KO or A<sub>1</sub>-A<sub>2A</sub> double KO mice were attenuated significantly ( $p=0.005$ ,  $p=0.017$ ,  $p=0.030$ , respectively, Holm's corrected  $p$ -values) in comparison to WT (Figure 1B). The attenuation appeared incomplete in all KO genotypes, with responses in KO mice during the plateau phase still significantly increased compared to their initial (day 1) response ( $p<0.05$ , Figure 1). Importantly, the effects of A<sub>1</sub> and A<sub>2A</sub> deletion on total AIMs were non-additive. In fact, for total AIMs, the A<sub>1</sub>-A<sub>2A</sub> double KO mice had higher levels than the A<sub>1</sub> KO mice at all time points.

Total AIMs were further divided into rotational and non-rotational AIMs subscales. Rotational AIMs comprised most (~90%) of total AIMs, and they were attenuated in all genotypes ( $p=0.005$ ,  $p=0.018$ ,  $p=0.018$ , respectively, Holm's corrected  $p$ -values), whereas non-rotational AIMs showed no difference that reached statistical significance compared to WT littermates.

### **Effect of Caffeine on L-DOPA-induced behavioral sensitization**

The mixed adenosine A<sub>1</sub>-A<sub>2A</sub> antagonist caffeine, at the low dose of 3mg/kg, significantly attenuated AIMs that developed in response to repeated L-DOPA administration ( $p=0.02$ , Figure 2B), and tended to reduce sensitized rotational responses ( $p=0.13$ , Figure 2A). The initial motor stimulating effect may confound the attenuation effect of caffeine on chronic contralateral rotations. Low dose of caffeine reduced rotational components of AIMs ( $p=0.03$ ; data not shown), but not non-rotational AIMs ( $p=0.22$ ). The discrepancy between rotation and AIMs recording is most likely due to the differences between these two measurements (Carta et al., 2006). An increase in contralateral rotations could be due to the anti-parkinsonian effect of caffeine rather than an increase in dyskinesia (Lane et al., 2006; Marin et al., 2006). Not surprisingly and consistent with our previous finding (Yu et al., 2006), the higher dose of caffeine (15 mg/kg) did not significantly attenuate either L-DOPA-induced rotations or AIMs ( $p>0.05$ , data not shown), possibly due to its general motor stimulant actions.

### **Modulation of striatal gene expression and residual dopamine**

In WT mice striatal PPE mRNA levels were significantly elevated on the lesioned side after chronic L-DOPA treatment (Figure 3), as demonstrated previously and consistent with the activation of PPE-expressing striatopallidal neurons with the development of LID in parkinsonian rodents and primates [e.g. review (Xu et al., 2005)]. This elevation of PPE was absent in all adenosine receptor KOs, which showed significantly lower striatal PPE mRNA ipsilateral to 6-OHDA lesioning after chronic L-DOPA treatment, compared to their WT littermates (Figure 3). The PPD on the lesioned

side tended to be reduced in comparison to the non-lesioned side (Student *t*-test,  $p=0.07$ ) in  $A_{2A}$  KO mice, consistent with our previous finding (Fredduzzi et al., 2002), in  $A_1$  KO ( $p=0.09$ ) and  $A_1$ - $A_{2A}$  KO ( $p=0.09$ ). However, the intact side of adenosine receptor KO mice showed no difference in PPE ( $p>0.05$  for all KOs mice) and PPD ( $p>0.05$  for all KOs mice, student *t*-test) in comparison with WT control, suggesting that L-DOPA treatment had no differential effect on gene expression in unlesioned striata across genotypes .

To address the possibility of any change of the prepeptide genes due to the gene KO itself, we also studied the baseline changes, if any, of PPE and PPD in the colonies of  $A_1$ ,  $A_{2A}$  and  $A_1$ - $A_{2A}$  double KO mice. We found there was no difference of these peptide gene expression among the different genotypes ( $p>0.05$ , one-way ANOVA and Student *t*-test, supplementary figure), consistent with previous finding (Chen et al., 1999).

Consistent with adenosine  $A_1$ - $A_{2A}$  receptor KO, low dose of caffeine (3 mg/kg) pretreatment tended to reduce the expression of PPE on the 6-OHDA lesioned side ( $n=6$ ,  $p=0.08$ , student *t*-test), corresponding to caffeine's attenuating effect on LID.

Genotype or pharmacological blockade did not affect the dopamine levels in the 6-OHDA-lesioned and contralateral (non-lesioned) striata (Table 1 and Table 2, respectively).



## Discussion

Genetic elimination of adenosine receptors – either A<sub>1</sub> or A<sub>2A</sub> or both – attenuated the development of AIMs and associated sensitized rotational responses to L-DOPA in a 6-OHDA lesion model of PD. Though we had previously demonstrated the A<sub>2A</sub> receptor-dependence of these phenomena (Xiao et al., 2006), our demonstration of a similar dependence on A<sub>1</sub> receptors prompted a complementary pharmacological test of the mixed A<sub>1</sub>-A<sub>2A</sub> caffeine in this model of LID. At a low dose (at the threshold of its motor stimulatory effect) caffeine also reduced AIMs. We also found that that induction of PPE, a marker of striatal (nigropallidal) neuron activity, was attenuated in the adenosine receptor KO striata. Finally, striatal dopamine levels were unaffected in KO or caffeine-treated mice, suggesting that A<sub>1</sub> and A<sub>2A</sub> receptors facilitate the maladaptive neuroplasticity of this LID model rather than the nigrostriatal lesion that is required for its full manifestation.

### The role of A<sub>1</sub> and A<sub>2A</sub> receptors in L-DOPA-induced dyskinesia

Earlier studies have shown that blockade of A<sub>2A</sub> receptors can improve symptoms of PD and decrease neurodegeneration (Chen, 2003; Chen et al., 2007). Here we show that LID can be reduced by A<sub>2A</sub> receptor blockade, consistent with our previous finding (Fredduzzi et al., 2002; Xiao et al., 2006). Despite our own evidence that A<sub>2A</sub> receptor depletion can confer neuroprotection in an MPTP model of PD, in others including the unilateral 6-OHDA-lesion model used here, we've observed no effects of A<sub>2A</sub> depletion on the neurochemical (dopamine content) or neuroanatomical (dopamine transporter ligand binding) indicators of dopaminergic neuron injury [(Fredduzzi et al., 2002; Xiao et

al., 2006); and Tables 1 and 2 in this paper]. Furthermore, caffeine was administered two weeks after the 6-OHDA lesioning in the present experiment, rendering unlikely the possibility that adenosine receptors contribute to LID indirectly through facilitation of neurodegeneration. Thus the reductions in LID reported here suggest a role for adenosine receptors in the neuroplasticity underlying LID.

The effects of blocking A<sub>1</sub> receptor in LID are less well understood, as there is clear evidence for multiple sites of presynaptic, as well as postsynaptic expression and actions of A<sub>1</sub> receptors in the striatum. For example, presynaptic striatal A<sub>1</sub> receptors inhibiting glutamate (Ambrosio et al., 1996; Ciruela et al., 2006), dopamine or acetylcholine release in the striatum and elsewhere might contribute to this complexity (Fredholm and Dunwiddie, 1988; Jin et al., 1993).

PPE expression in the 6-OHDA-lesioned striata was elevated relative to the intact side after L-DOPA-treatment as expected, likely reflecting overactivation of the striatopallidal pathway in LID [reviewed in (Xu et al., 2005)]. The increased PPE expression was attenuated in adenosine receptor KO compared to WT mice, consistent with previous findings that blockade of adenosine receptor reverses the elevated gene expression of PPE in lesioned striatum (Aoyama et al., 2002). Together these studies suggest that the so-called indirect pathway (in which A<sub>2A</sub> receptor-expressing striatopallidal neurons reside) plays a crucial role in the modulation of LID by adenosine receptors.

### **A<sub>1</sub> and A<sub>2A</sub> receptors interaction in L-DOPA-induced dyskinesia**

Although the attenuation of LID was partial in both the A<sub>1</sub> and A<sub>2A</sub> KO mice, there was no clear additivity or synergy of their attenuating effects on LID. In seeming contrast, the combination of A<sub>1</sub> and A<sub>2A</sub> antagonists by (Karcz-Kubicha et al., 2003) produced an additivity of their individual motor-activating effects. Adenosine receptor mechanisms and hence their interactions in acute motor activation likely differ from those in LID. Similarly, less than additive effects of D<sub>1</sub> and D<sub>2</sub> dopamine receptor antagonists have been reported on complex adaptive behaviors, whereas acute effects of these drugs in combination are typically at least additive on motor activity (Schneider et al., 1991). In LID A<sub>1</sub> and A<sub>2A</sub> receptors may have sufficiently redundant effects in parallel to preclude an additional effect of blocking one receptor after the other has been inactivated. Alternatively, A<sub>1</sub> and A<sub>2A</sub> receptor roles in LID may occur sequentially – either when colocalized to a single cell or when interacting in different cells at a network level [(e.g. review by (Lopes et al., 2002; Xu et al., 2005)]. The lack of clear additivity observed here may also reflect the chronicity of complete, lifelong inactivation of A<sub>1</sub> and/or A<sub>2A</sub> receptors in constitutive KO mice, compared to the acuity of interaction with most pharmacological treatments.

Furthermore, the similar attenuations of LID in adenosine receptor KO and after adenosine antagonist (caffeine) application in adult mice support a contention that the observed KO phenotype reflects actions of A<sub>1</sub> and A<sub>2A</sub> receptors in the adult brain. Though the complete elimination of a receptor subclass as occurs in the homozygous mutant state can offer an incisive initial assessment of its biological role, it may not be the best predictor of pharmacotherapy. Effects of a receptor antagonist like caffeine are

mediated by blockade of only a subset of its receptors, such that a subtler or even distinct phenotype of a heterozygous compared to homozygous KO mouse may be most instructive. Indeed we have recently demonstrated that some behavioral characteristics of doubly ( $A_1$  and  $A_{2A}$ ) heterozygous mice can match some motor effects of repeated caffeine exposure (Yang et al., 2009).

### **Translational significance**

The results of this study suggest that both  $A_1$  as well as  $A_{2A}$  receptor antagonists could be useful therapeutically to lower the risk of LID in PD patients. The results also raise the possibility that blocking both  $A_1$  and  $A_{2A}$  receptors simultaneously, as occurs with caffeine use, could provide a prophylaxis against LID development similar to that of a specific  $A_1$  or  $A_{2A}$  antagonist alone. Currently caffeine (Postuma, 2009) and multiple specific  $A_{2A}$  receptor antagonists (Xu et al., 2005; Biogen Idec. Cambridge, 2008; Black, 2008; Schering-Plough, 2009) are undergoing randomized clinical trials for possible symptomatic benefits in later stages of the disease after dopaminergic treatment has been initiated. Our findings lend further support to the emerging rationale for investigating the early adjunctive use of adenosine antagonists in PD as potential disease-modifying therapy (Ravina et al., 2003; Schwarzschild et al., 2006).

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## Figure legends

**Figure 1.** Attenuation of contralateral rotations and abnormal involuntary movement (AIM) in  $A_1$ ,  $A_{2A}$  and  $A_1$ - $A_{2A}$  double KO mice compared to WT. Mice were treated daily with L-DOPA (2 mg/kg, i.p.) in combination with benserazide (2 mg/kg, i.p.) for 3 weeks. **A**, Contralateral turns in mice treated with L-DOPA. **B**, Total AIMs in mice treated with L-DOPA. Significant differences in the total AIMs in the plateau phase were observed between the WT (n=12) and the  $A_1$ KO (n=9),  $A_{2A}$ KO (n=8) or  $A_1$ - $A_{2A}$  KO (n=8) groups ( $p < 0.05$  for each comparison, Holm's correction). The attenuation of AIMs during the plateau phase significantly increased compared to day 1 response, except day 14 and day 17 for  $A_1$  KO.

**Figure 2.** Attenuation of abnormal involuntary movements (AIMs) by a low dose of caffeine. Mice were treated daily for 18 days with L-DOPA (2 mg/kg, i.p. in combination with benserazide 2 mg/kg, i.p.) 10 mins after i.p. administration of saline or caffeine (3mg/kg). **A**, Contralateral turns in mice pretreated with saline or caffeine ( $p = 0.13$ , comparing caffeine to saline in the plateau phase). **B**, Total AIMs in mice pretreated with saline or caffeine ( $p < 0.05$ , comparing caffeine to saline in the plateau phase).

**Figure 3.** Reduction of PPE mRNA in lesioned striata of adenosine receptor knockout mice following chronic L-DOPA treatment. PPE mRNA levels (**A**) in the 6-OHDA-lesioned striata were quantified as optical density (OD) at the mid-striatum level and expressed as a ratio to OD of the contralateral (unlesioned) striatum (**B**). Chronic treatment with L-DOPA reduced the 6-OHDA-induced increase in striatal PPE levels in

A<sub>1</sub> KO (n=4), A<sub>2A</sub> KO (n=5) and A<sub>1</sub>-A<sub>2A</sub> KO (n=4) mice (\*p<0.05; Student's *t*-test compared with the WT group, n=5).



Table 1. Neurochemical measure of nigrostriatal innervation in wild-type and adenosine receptor KOs mice chronically treated with L-DOPA after a unilateral 6-OHDA lesion.

Genotype	DA (pm/mg tissue)	DOPAC (pm/mg tissue)
<b>WT (n=12)</b>		
Ipsilateral (lesioned)	4.2 ± 1.4*	0.0 ± 0.0*
Contralateral (intact)	143.6 ± 7.5	11.3 ± 1.7
<b>A<sub>2A</sub> KO (n=9)</b>		
Ipsilateral (lesioned)	4.3 ± 3.0*	0.7 ± 0.5*
Contralateral (intact)	129 ± 10	15.9 ± 7.4
<b>A<sub>1</sub> KO (n=8)</b>		
Ipsilateral (lesioned)	6.9 ± 3.0*	0.4 ± 0.4*
Contralateral (intact)	120 ± 14	9.7 ± 0.9
<b>A<sub>1</sub>.A<sub>2A</sub> KO (n=7)</b>		
Ipsilateral (lesioned)	10.4 ± 8.2*	1.2 ± 0.5*
Contralateral (intact)	118 ± 18	9.6 ± 0.9

\*  $p < 0.05$  versus respective contralateral (intact) striatum. The dopamine level in the intact (right) side between wild-type and three other genotypes was not significantly different ( $p > 0.05$ ).

Table 2. Neurochemical measure of nigrostriatal innervation in saline and caffeine (3mg/kg) pretreated mice daily treated with L-DOPA after a unilateral 6-OHDA lesion

Treatment	DA(pm/mg tissue)	DOPAC(pm/mg tissue)
<b>Saline (n=6-7)</b>		
Ipsilateral (lesioned)	$0.4 \pm 0.2^*$	$1.0 \pm 0.8^*$
Contralateral (intact)	$90.8 \pm 14.5$	$21.8 \pm 14.9$
<b>Caffeine (n=6)</b>		
Ipsilateral (lesioned)	$4.6 \pm 2.6^*$	$0.6 \pm 0.4^*$
Contralateral (intact)	$103.1 \pm 17.6$	$23.4 \pm 15.1$

\*  $p < 0.05$  versus respective contralateral striatum. The dopamine level in the intact (contralateral) side between saline and caffeine groups was not significantly different ( $p > 0.05$ ).

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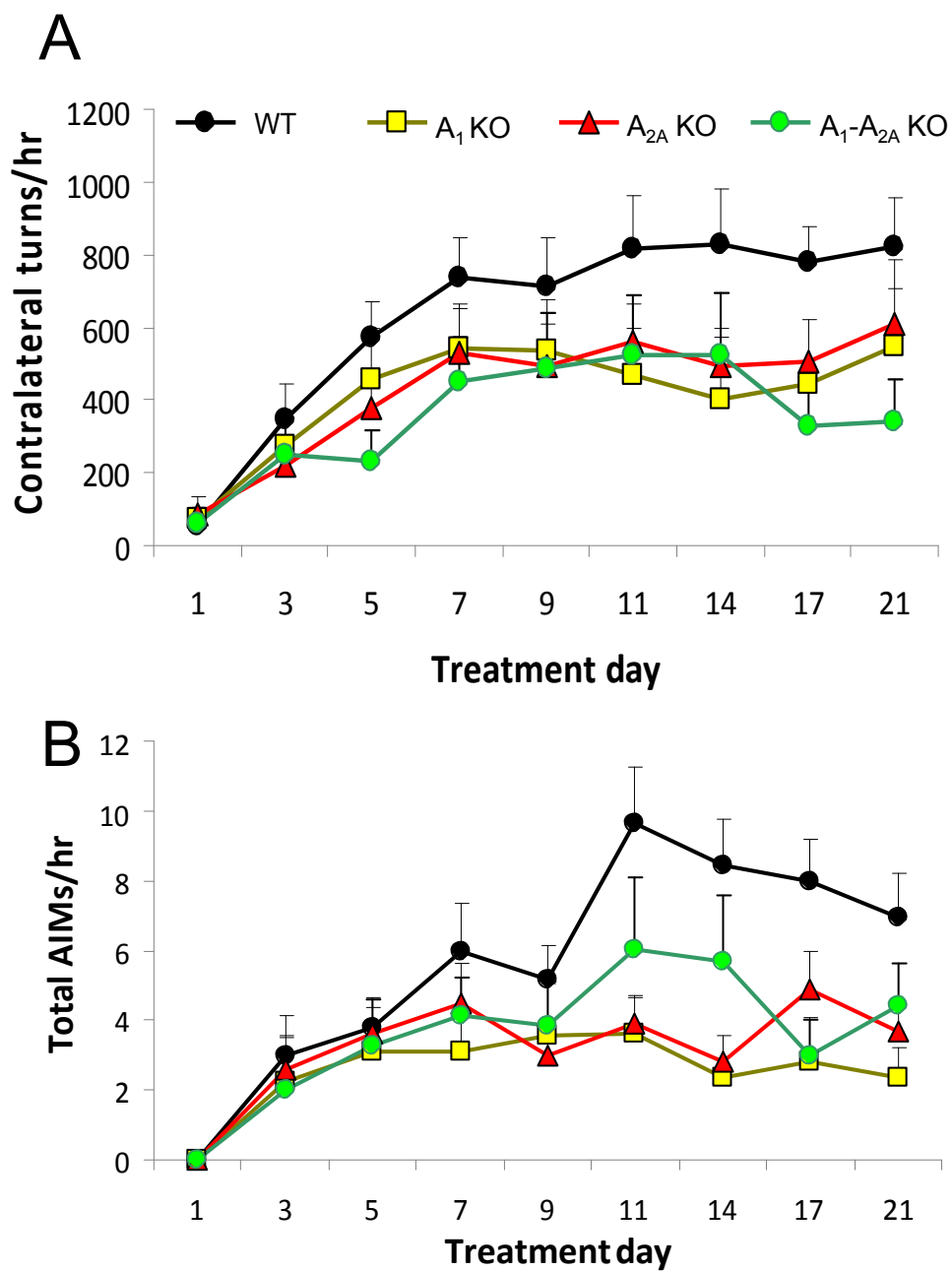


Figure 1  
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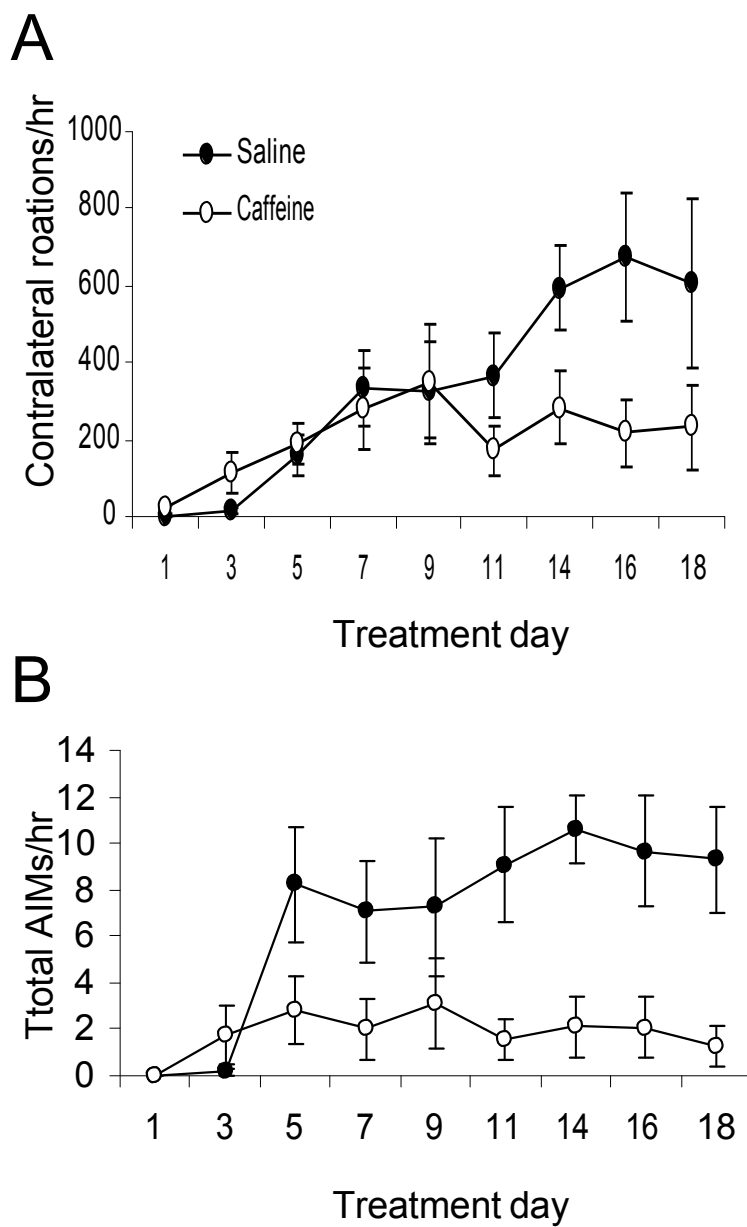


Figure 2  
Xiao et al, 2009



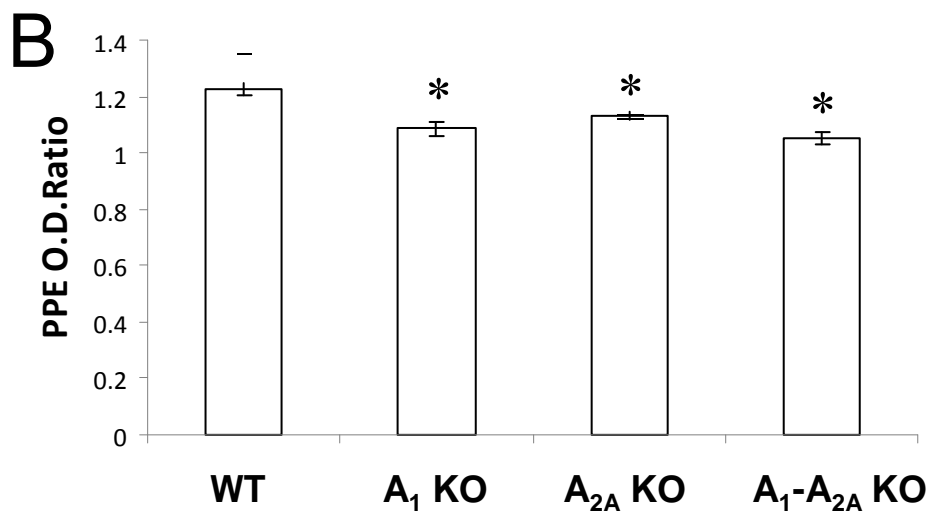
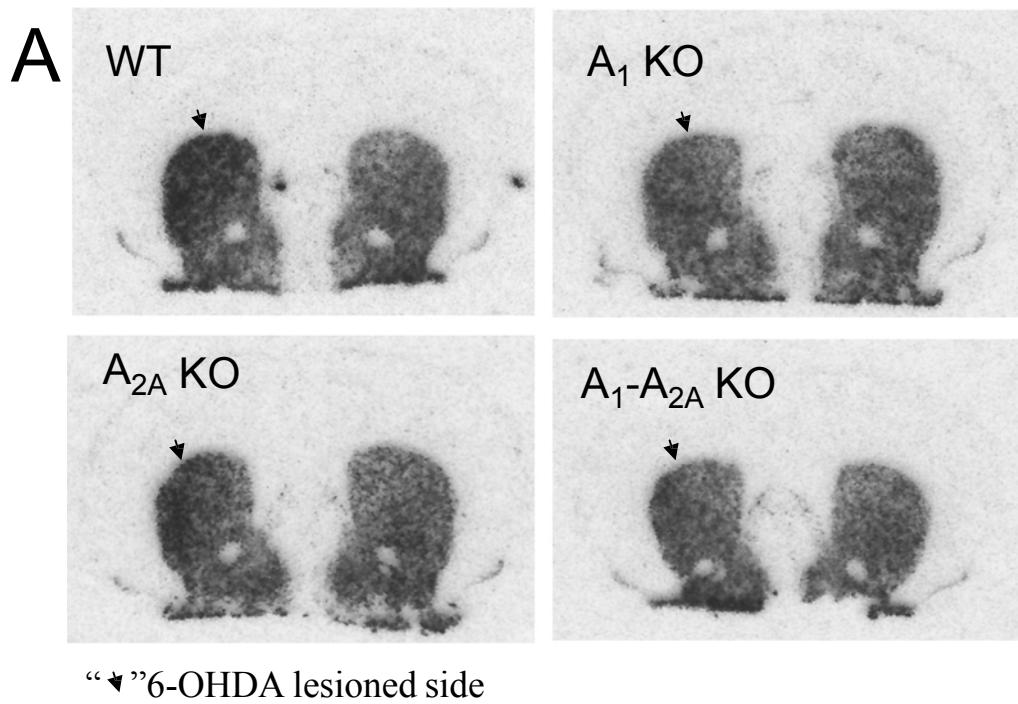


Figure 3  
Xiao et al, 2009